THE OCCURRENCE OF SALMONELLA TYPES IN AUSTRALIA[1]. 6.

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The cultures described in this paper came almost exclusively from an outbreak of gastro-enteritis which Ferris and Hertzberg (1945) reported in military personnel stationed on the Atherton Tableland, Queensland. Under the tentative name *S. atherton,* Atkinson, Woodroofe and Macbeth (1947) recorded 31 strains from this outbreak, and in the present paper give an account of the detailed investigation of these strains and one South Australian strain found subsequently. Serologically all strains appeared identical but failed to conform to any Salmonella antigenic formula. They possessed the H factor z_4 which occurs in several Salmonellas and also in many members of a group of paracolon bacteria described by Edwards, Cherry and Bruner (1943) and later called the Arizona group (Edwards, West and Bruner, 1947). However, the *atherton* cultures possessed none of the recognized Salmonella O factors but their somatic antigens closely resembled those of a member of the Arizona group known as NJ4, which did not include z_4 among its flagellar antigens. Our strains therefore differed in their flagellar factors from NJ4 and did not appear to be identical with any of the Arizona group. Unlike this group, which occurred mainly in snakes, birds and egg powder, our strains appeared to be human pathogens. Ferris and Hertzberg (1945) deduced that they were the cause of the disease in their outbreak as these organisms, but none of the known pathogens, were isolated from the majority of patients, they disappeared after recovery, and O agglutinins for them were detected in the blood of sufferers but not in the blood of others. The South Australian strain of *S. atherton* was isolated from the faeces of a child with diarrhoea and rectal haemorrhage. If *S. atherton* were acceptable as a Salmonella its O antigen would be new and its H antigens, which were very like those of *S. cerro,* could be represented as $z_4, z_{23}, \ldots$. After our work had been completed we received from Dr. E. Seligmann a culture of a new Salmonella, *S. waycross* (Seligmann and Saphra, 1948) which was represented by one strain derived from the urine of a case of chronic cystitis. This organism appeared to be antigenically similar to *S. atherton* but differed from the majority of our strains in dulcitol fermentation. Like

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S. atherton, Dr. Seligmann's organism came from human material but its connection with the pathological condition was not examined. Although antigenically *S. waycross* and *S. atherton* appeared identical, the name *S. atherton* is retained in this paper to distinguish our strains and establish their identity with *S. waycross*. Subsequently the name *S. atherton* should no longer be used.

EXPERIMENTAL.

Cultural and Biochemical Reactions.

Thirty strains of *S. atherton* including 29 from the Atherton outbreak and one from South Australia were used in these investigations. All were motile Gram-negative bacilli which grew freely on nutrient agar or broth. In peptone water at 37° C. they rapidly produced acid and gas from glucose, maltose, mannitol, trehalose, arabinose, rhamnose and xylose but did not attack lactose, sucrose, salicin and inositol during 30 days. Acid and gas were also produced in dulcitol by all except the South Australian strain (Cronin) which failed to attack this substrate in 30 days. All failed to liquefy gelatine in 60 days at 22° C. or to form indol in peptone water during 5 days at 37° C. Abundant H_2S was detected from nutrient agar cultures in 24 hours at 37° C.

In view of the serological relationship between *S. atherton* and the Arizona group which slowly liquefied gelatine and, with a few exceptions, slowly fermented lactose, the effect of our strains upon lactose and gelatine was extensively investigated. Preliminary investigation (Atkinson, 1945) had suggested that some of the strains of *S. atherton* slowly liquefied gelatine and slowly formed slight acid in sucrose and lactose. However, these reactions were not confirmed in later tests and were probably due to the low quality of many of the war-time chemicals. Using post-war analytical chemicals repeated tests in 1 p.c. lactose peptone water in containers sealed with paraffin, as recommended by Peluffo, Edwards and Bruner (1942), showed no fermentation during 60 days at 37° C. Under the same conditions representative Arizona strains, 36608, Hudson, S-39 and NJ4 (kindly provided by Dr. P. R. Edwards) produced acid and usually gas after two to forty days, and *S. cerro* and *S. dusseldorf*, which contained the z_4 flagellar factor, produced no change. Edwards, Cherry and Bruner (1943) found that serial passage every five days in 5 p.c. lactose broth often increased the rate of fermentation of lactose by their paracolon bacteria, although one strain remained negative throughout. All the strains of *S. atherton* were therefore subcultured serially in 5 p.c. lactose peptone water with five to eight days between each subculture. Five series were made and all cultures were observed for 60 days but no acid or gas was detected. *S. cerro* and *S. dusseldorf* were passed in the same way through the 5 p.c. lactose medium and showed no fermentation, whereas the Arizona strains 36608, Hudson S-39 and NJ4 all produced acid and gas in three days at the fourth transfer. The same group of organisms was tested on several occasions for liquefaction of gelatine. The cultures were incubated at 22° C. and observed for about three months. No liquefaction was detected with any strain of *S. atherton* or with *S. cerro*, *S. dusseldorf* or Hudson but the Arizona types 36608, S-39 and NJ4 produced liquefaction in 16 to 50 days. The Arizona group was described by Edwards *et al.* (1947) as not fermenting dulcitol or utilizing d-tartrate, whereas our strains of *S. atherton*, with the exception of the South Australian strain which was dulcitol negative, readily attacked both these substrates. These reactions were confirmed on several occasions with the group of cultures used in the previous tests. *S. atherton* strains with the exception noted, *S. dusseldorf* and *S. cerro* produced acid and gas from dulcitol in 24 hours at 37° C., but the Arizona types 36608, Hudson, S-39 and NJ4 produced no change in 20 days. Furthermore all the *atherton* cultures, *S. dusseldorf* and *S. cerro*

produced acid in 24 hours at 37° C. in the phenol red tartrate agar of Jordan and Harmon (1928) but the Arizona cultures 36608, Hudson, S-39 and NJ4 showed no acidity after three days.

S. waycross arrived after this work was completed. Its fermentative activities were compared with those of a few representative strains of *S. atherton* and the Arizona group. It resembled *S. atherton* in rapidly fermenting glucose, maltose, mannitol, trehalose, arabinose, xylose and rhamnose, in failing to attack salicin or inositol and in producing acid in Jordan's tartrate medium in 24 hours. It resembled the Arizona group and our South Australian strain of *S. atherton* in not fermenting dulcitol. These results confirmed the reactions which Seligmann and Saphra (1948) recorded for this organism.

Serological Investigations.

The general methods used here followed those described either by Edwards and Bruner (1940) or Kauffmann (1941).

In slide tests with Salmonella O antisera, representing all the O antigens except XLI and XLII, then undescribed, our strains of *S. atherton* either failed to react or produced only a trace of agglutination with a few sera. In no case was the agglutination sufficiently marked to consider allocating the organisms to the corresponding O group. O antisera were prepared against the Arizona types known as Arizona, CDAI 125, CDAI 44, S-39, Calif. 166, CDAI 184, CDAI 434, N99, 36608, Ore. 181, Sp 21, NJ4, Hudson, Pe 139, So 34, So 5, Pe 145, Pe 148, Pe 167, So 116, all of which were kindly supplied by Dr. P. R. Edwards. In slide tests with these Arizona O antisera the *atherton* cultures gave strong agglutination with NJ4 serum, weak agglutination with CDAI 184 and Ore. 181 serum and no reaction with any other serum. In tube tests done on several occasions using alcoholized suspensions of NJ4 and *S. atherton* (strain Burns), NJ4 O antiserum agglutinated both suspensions to the same titre of 1/1,280. *S. atherton* O antiserum was prepared against each of two strains, Burns and Gibbs. In slide tests the Burns or Gibbs serum strongly agglutinated all other *atherton* cultures and NJ4, but did not react with any of the other Arizona strains mentioned above. In tube tests Burns serum agglutinated suspensions of three *atherton* strains (Burns, Gibbs and Tregear) and NJ4 to the same titre of 1/1,280. In absorption tests NJ4 organisms removed all agglutinins for *S. atherton* from Burns O antiserum, and *S. atherton* (strain Burns) removed all agglutinins for NJ4 from the NJ4 O antiserum. The heat stable O antigens of *S. atherton* were therefore considered identical with those of NJ4.

In tube tests with a comprehensive range of Salmonella H antisera, H suspensions of all strains of *S. atherton* agglutinated only with *S. cerro* (z_4, z_{23}) antiserum. In further tests *S. atherton* readily agglutinated in H antisera prepared against *S. dusseldorf* (z_4, z_{24}) or *S. tallahassee* (z_4, z_{32}). *S. atherton* therefore possessed the H factor z_4. No second phase was detected after several serial passages in semi-solid agar containing *S. cerro* antiserum.

To detect the H factors z_{23} or z_{24} likely to be present with z_4, all strains of *S. atherton* were tested with absorbed sera specific for these factors and prepared in the following way (Edwards and Bruner, 1940):

For factor z_{23}—*S. cerro* serum absorbed with *S. dusseldorf*.

For factor z_{24}—*S. dusseldorf* serum absorbed with *S. cerro*.

Agglutination occurred with z_{23} serum only.

To investigate further the relationship between the flagellar antigens of *S. atherton*, *S. cerro*, *S. dusseldorf* and *S. tallahassee*, H antisera were prepared against two strains, Burns and Gibbs, of *S. atherton* and cross absorption tests performed. The two *atherton* strains were identical. *S. atherton* (Burns) completely removed H and O agglutinins from Gibbs serum and *S. atherton* (Gibbs) completely removed H and O agglutinins from Burns serum. Burns serum

absorbed with *S. dusseldorf* or *S. tallahassee* still agglutinated *S. atherton* H suspensions in high dilution. *S. dusseldorf* or *S. tallahassee* serum absorbed with *S. atherton* (Burns) was reduced only slightly in H titre for the homologous organism. Absorption of Gibbs or Burns serum with *S. cerro*, or *S. cerro* serum with *S. atherton* (Gibbs or Burns) removed the greater part of the H agglutinins for the homologous organism. In such absorbed serum however, residual agglutinins usually remained at low titre (1/100–1/200) for the homologous organism. The absorptions were repeated many times and occasionally no agglutinins for the homologous organism were detected at 1/100 which was the lowest dilution tested. These results contrasted with those obtained in absorptions of Burns or Gibbs serum with *S. atherton* in which all agglutinins were readily removed. We therefore concluded that the *atherton* strains were antigenically identical and their H antigens were closely related to, but not quite identical with, those of *S. cerro*.

In the Arizona group, according to Edwards, West and Bruner (1947), many members contain the Salmonella H factor z_4 associated in some cases with z_{23}. In case our strains were more closely related to these organisms in their flagellar antigens *S. atherton* was compared with representative z_4 and other types of the Arizona group. Those selected included Arizona (z_4, z_{23}, z_{26}), 36608 (z_4, z_{23} ...), CDAI 184 (z_4 ...), Pc 143 (z_4 ...), N178 (z_4, z_{23} ...), S-39 (z_4, z_{24} ...), Sp 21 (z_4 ...), Hudson (z_4, z_{23}, z_{26}), NJ4 (g ...), N99 (g ...), Pc 107 (no Salmonella H antigens) and Ore. 181 (z_{29} ...). Typical results of agglutination tests are given in Table 1 including *S. cerro* for comparison with the Arizona group.

TABLE 1.

Agglutination tests with S. atherton (*Burns or Gibbs*) *antiserum and H suspensions of members of the Arizona group.*

H suspensions	*S. atherton* antiserum	
	Burns	Gibbs
S. atherton (Burns)	5,000	6,000
S. atherton (Gibbs)	5,000	6,000
S. atherton (Tregear)	5,000	0
S. cerro	5,000	6,000
Arizona	1,000	3,000
Hudson	1,000	0
36608	500	800
S-39	300	800
CDAI 184	200	400
Sp 21	200	400
NJ4	—	0
N99	—	0
Pc 107	—	—
Ore. 188	—	0

The figures are the reciprocals of the highest dilutions producing H agglutination in 2 hours at 55° C.

— = no agglutination at 1/40. 0 = not tested.

These results indicated that, in the Arizona group, the H antigens of Arizona and Hudson most closely resembled those of *S. atherton*, but all other z_4 members tested were related in a minor degree only to *S. atherton*. Those members without the factor z_4 showed no relation at all to *S. atherton*.

With *S. atherton* (Gibbs or Burns) antiserum absorptions were done with various Arizona strains. Burns serum remained practically unaltered in titre for an H suspension of *S. atherton* (Burns) after absorption with CDAI 184, S-39 or Sp 21, but the titre was reduced after absorption with Arizona, 36608 or Hudson. Similarly Gibbs serum was reduced in H titre by absorption with Arizona or 36608 but changed very little after absorption with S-39, CDAI 184 or Sp 21. Typical results for these tests are presented in Table 2.

TABLE 2.

Agglutination tests with S. atherton (*Burns or Gibbs*) *H suspensions and* S. atherton (*Burns or Gibbs*) *antiserum after absorption with various members of the Arizona group.*

Absorbing organism	*S. atherton* antiserum	
	Burns	Gibbs
Arizona	800	800
Hudson	800	0
36608	800	800
S-39	2,000	3,000
CDAI 184	3,000	2,000
Sp 21	2,000	2,000
None	3,000	3,000

The figures are the reciprocals of the highest dilutions producing H agglutination of an H suspension of *S. atherton* (Burns or Gibbs according to the serum under test) in 2 hours at 55° C. No agglutination occurred at 1/100 with the absorbing organism.

0 = test not done.

Thus none of the Arizona group tested here completely absorbed the H agglutinins from *S. atherton* antiserum and therefore did not possess H antigens identical with *S. atherton*. Absorptions were also performed with Arizona antiserum and *S. atherton* (Burns or Gibbs). Before absorption Arizona antiserum agglutinated H suspensions of Arizona or *S. atherton* (Burns or Gibbs) to a dilution of 1/3,000. After absorption the serum failed to agglutinate *S. atherton* at 1/100 but still agglutinated Arizona to a dilution of 1/800. *S. atherton* thus possessed only a part of the H complex of Arizona, which occurred in about one third of the 373 strains of the Arizona group described by Edwards, West and Bruner (1947).

On these results, *S. atherton*, if accepted as a Salmonella, would require a new symbol for the diagnostic O factor identical with that of NJ4, which already has a designation in the Arizona group, and the H factors would be represented by the same symbols for *S. cerro*, z_4, z_{23} .... However, these H factors are also found in many members of the Arizona group and Edwards, West and Bruner (1947) have suggested antigenic formulae to distinguish the Arizona types. They gave numbers to the diagnostic antigens. In their scheme NJ4 had the O factor 13 and the H factors, designated as z_4, z_{23} and z_{25} in *S. cerro*, were called paracolon factors 1, 2 and 4. In the Arizona group all the diagnostic factors of our *atherton* strains occur and our organisms could therefore be represented in this group by the antigenic formula Ar. 13:1, 2, 4, which did not occur among the 373 Arizona types so far described by Edwards *et al.* (1947). This unusual combination of antigens and the biochemical reactions of the *atherton* strains do not support their inclusion in the Arizona group.

This work was completed when we received the culture of *S. waycross*. Its close relation to *S. atherton* was immediately apparent in its strong agglutination in both slide and tube tests

with *atherton* O antiserum. Its H suspensions were agglutinated by *atherton* and other z_4 antisera in high dilution. H antisera were prepared against *S. waycross* and absorbed with *S. atherton* (Burns, Gibbs or Cronin). After absorption the serum at 1/100 failed to agglutinate an H suspension of *S. waycross*. *S. atherton* (Burns or Gibbs) H antiserum after absorption with *S. waycross* failed at a dilution of 1/100 to agglutinate H suspensions of *S. atherton* (Burns or Gibbs). *S. waycross* O antiserum absorbed with *S. atherton* (Burns, Gibbs or Cronin) failed at a dilution of 1/10 to agglutinate *S. waycross*. O antiserum for *S. atherton* (Burns or Gibbs) absorbed with *S. waycross* failed at 1/10 to agglutinate *S. atherton*. These absorptions were as clear-cut and complete as those performed among the *atherton* strains themselves. *S. waycross* was therefore considered to have an antigenic formula identical with that of *S. atherton*. Seligmann and Saphra (1948) in their description of *S. waycross* pointed out that its O antigen was new to the Salmonella group and was to be represented by the new symbol XLI. The antigenic formula for *S. atherton* and *S. waycross* would then be XLI: z_4, z_{23} .... They differed from each other only in action on dulcitol and if the two names were retained on the basis of this minor difference our South Australian strain would be identified as *S. waycross*.

Habitat and Pathogenicity.

S. atherton appears to be a rare type. Only one epidemic, from which our strains came, has been described as due to this organism (Ferris and Hertzberg, 1945). From this single epidemic, however, about 30 strains were obtained and allowed adequate characterization of the organism. The epidemic, in which no other recognized pathogen was detected, occurred in Queensland, but no further strains have been received from that State. The type of illness varied from mild, acute gastro-enteritis with vomiting, diarrhoea and fever to almost inapparent infections detected only by direct questioning of contacts. Ferris and Hertzberg (1945) tested the serum of 26 patients 11 to 14 days after the onset of illness and obtained agglutination of an O suspension of *S. atherton* with a titre of 1/300 for some sera. Sera from 100 normal people failed to agglutinate the same suspension. From the stools of some cases *S. atherton* was isolated up to 20–40 days after the illness commenced. The South Australian strain was isolated at the Adelaide Children's Hospital from the faeces of a child who was admitted with a history of having passed small blood-stained motions every 20 minutes for the last 24 hours. This patient quickly improved but the Salmonella was detected in the stools for about a month. No other pathogens were found. About a year earlier this patient had been admitted with haemorrhage per rectum and abdominal pain. A duodenal ulcer was found and treated for several months in the hospital during which time recurrences of bleeding per rectum occurred. The patient recently had another recurrence of rectal bleeding.[2] Undoubtedly *S. atherton* is a human pathogen producing gastro-enteritis which, judging from the Queensland cases, may be milder than the usual Salmonella gastro-enteritis. No animal reservoir of the organism has yet been discovered.

Groups of mice were injected intraperitoneally with *S. atherton* (Burns and Gibbs) in doses varying between 100×10^6 and 500×10^6 organisms. The cultures used were smooth as judged by the colony form, growth in broth, trypaflavine test and stability in 3·6 p.c. and 6·8 p.c. saline. With the Gibbs strain one out of five mice inoculated with 125×10^6 organisms and two out of five mice inoculated with 250×10^6 organisms died within 48 hours and *S. atherton* was recovered from the heart blood. The other mice were still alive after 14 days.

[2] We are indebted to Dr. H. G. Rischbieth, Medical Superintendent of the Adelaide Children's Hospital for providing this history.

One of each group was killed and *S. atherton* was recovered from liver and spleen. With *S. atherton* (Burns) four out of four mice receiving 500×10^6 organisms and two out of four mice receiving 250×10^6 organisms died within three days. The remaining two mice died within 12 days. From all these mice *S. atherton* was recovered from the heart blood.

DISCUSSION.

Thirty strains of a Salmonella tentatively named *S. atherton* (Ferris and Hertzberg, 1945) were described in this paper and provided data adequate for its characterization. Although this organism possessed none of the then recognized Salmonella O antigens its H antigens were very like those of *S. cerro* and could be represented by the symbols $z_4, z_{23}, \ldots$. The factor z_4, with z_{23}, or other factors, occurs in a number of Salmonellas. On the other hand it occurs, with or without z_{23}, extensively in the Arizona group of paracolon bacteria of Edwards, West and Bruner (1947). The member of this group called Arizona was originally placed by Kauffmann (1941) in the Salmonella group under the name *S. arizona*. Its O antigen was different from that of any recognized Salmonella and was allotted the symbol XXXIII; but its H antigens were related to those of *S. cerro* and contained the Salmonella factors z_4 and z_{23}. Its biochemical reactions did not conform to those of the Salmonella group as it slowly fermented lactose and liquefied gelatine. However, it was isolated from natural infections in reptiles and, in spite of its aberrant biochemical reactions, it was included with the Salmonellas. No recognized Salmonella has subsequently been found containing the O antigen XXXIII and Edwards, West and Bruner (1947) quote a personal communication from Kauffmann in 1945 in which he agreed that *S. arizona* and related types be withdrawn from the Salmonella schema and regarded as paracolon bacteria, of which the Arizona group formed a subdivision. The problem of the classification of *S. arizona* has been outlined here because it involved some considerations similar to those involved in classifying *S. atherton*. Like that of Arizona, the O antigen of *S. atherton* was new to the Salmonella group, but unlike the Arizona O antigen it was already known, as it had been described in a paracolon organism, NJ4 of the Arizona group. However, the H antigens of *S. atherton* seemed more closely related to those of *S. cerro* than to those of Arizona and related paracolons although all contained the factor z_4 with or without z_{23}. In fact neither the complex z_4, z_{23}, z_{25} representing *S. cerro* (but contracted to z_4, z_{23}, ... in the diagnostic schema) nor the factor z_{25} occurred in any Arizona type found among the 373 cultures of Edwards, West and Bruner (1947). In biochemical reactions also *S. atherton* resembled the Salmonellas rather than the Arizona group in failing to liquefy gelatine or ferment lactose, in breaking down d-tartrate and (with the exception of the South Australian strain) in rapidly attacking dulcitol. The occurrence of *S. atherton* O antigen in the

Arizona paracolon type NJ4 in no way invalidates this component as a Salmonella antigen but simply extends the already wide list of paracolon bacteria with Salmonella antigens. *S. atherton* also resembles the Salmonellas in producing gastro-enteritis in man whereas few members of the Arizona group were obtained from human material. Thus on all characteristics, biochemical, antigenic and pathogenic, *S. atherton* conforms for inclusion in the Salmonella group. All our investigations were completed when *S. waycross* was described by Seligmann and Saphra (1948). This organism proved to be antigenically identical with *S. atherton* by routine methods and thus both organisms would be represented by the formula XLI; z_4, z_{23}, ...; −. The single strain of *S. waycross* differed from the Queensland group of strains of *S. atherton* in fermentation of dulcitol in which it resembled our South Australian strain. This difference seems insufficient to retain *S. waycross* and *S. atherton* as two distinct types. However, in Group C_1 of the Kauffmann-White schema three organisms are separately named with the same antigenic formula. They are *S. paratyphi C*, *S. cholerae-suis* and *S. typhi-suis*, all with the formula VI, VII; c; 1, 5. *S. paratyphi C* may have Vi antigen also but in its absence is identical with the others. Wilson and Miles (1946) call these types "fermentative varieties" which are revealed only by small differences in biochemical reactions. These authors doubt the significance of such types although they point out that some correlation has been observed between these differences and natural habitat or pathogenicity. However, the differences between these types are much greater than those by which *S. atherton* and *S. waycross* differ. In fact variation in dulcitol fermentation occurs among strains of numerous types. Also both *S. atherton* and *S. waycross* were isolated from human material but the dulcitol-negative cultures (our South Australian strain and the American strain of *S. waycross*) were not derived from typical gastro-enteritis. The American strain came from a patient with chronic cystitis with fever, and the South Australian strain occurred in a patient with recurrent rectal haemorrhage not regularly associated with the presence of pathogens. On the other hand the dulcitol-positive strains seemed to have been the causative agents of the gastro-enteritis in the Queensland epidemic of Ferris and Hertzberg (1945). However, such differences between these fermentative varieties must await confirmation by the discovery of further strains and examination of their origin. Determination of dulcitol fermenting powers may have value in epidemiological work as all strains from our one epidemic were dulcitol-positive while the other single strains were dulcitol-negative.

For the various reasons given, the name *S. atherton* will no longer be used and our strains described previously under this title will subsequently be referred to as *S. waycross*.

SUMMARY.

Thirty strains of a Salmonella, which had tentatively been named *S. atherton*, have been studied. Twenty-nine strains came from an epidemic of gastro-enteritis in Queensland and one strain from a case of diarrhoea and rectal haemorrhage in South Australia. All strains gave biochemical reactions typical of the Salmonella group. Trehalose and arabinose were rapidly fermented but inositol was not attacked. All except the South Australian strain rapidly fermented dulcitol. The South Australian strain failed to attack dulcitol.

The O-antigen of *S. atherton* was identical with that of the Arizona paracolon type NJ4. *S. atherton* was monophasic and the flagellar antigens were almost identical with those of *S. cerro* and could be represented as z_4, z_{23}, ...

S. atherton was antigenically identical with the only available strain of a newly described type, *S. waycross*. *S. atherton* therefore has the antigenic formula XLI; z_4, z_{23}, . . . of *S. waycross* and the name *S. atherton* is no longer to be used.

The one strain of *S. waycross* resembled our South Australian strain and differed from our Queensland strains in failing to ferment dulcitol.

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THE OCCURRENCE OF SALMONELLA TYPES IN AUSTRALIA[1]

7. TWO NEW SALMONELLA TYPES, *S. MOROTAI* AND *S. BALL.*

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The first of the two new Salmonella types to be described in this paper was represented by two strains obtained from the faeces of two patients in an outbreak of food-poisoning involving about ten people in a small army unit at Morotai. The strains were isolated by Major A. A. Ferris and sent to us for typing. We suggest that the name *S. morotai* be given to this type whose suggested antigenic formula is XVII; *l, v* . . . ; 1, 2 . . . . The second new type was represented by a single strain isolated from the faeces of an adult with gastro-enteritis in the Royal Adelaide Hospital. The name suggested for this type is *S. ball* and the suggested antigenic formula is I, IV, XII; *y; e, n, x.*

The experimental work upon which the identification of *S. morotai* and *S. ball* was based is given in the following sections.

EXPERIMENTAL.

Cultural and Biochemical Reactions.

The two strains of *S. morotai* and the single strain of *S. ball* were motile Gram-negative bacilli growing freely upon nutrient broth or agar. They produced abundant H_2S but failed to form indol. They did not liquefy gelatine in 14 days at 22° C. In peptone water they rapidly produced acid and gas from glucose, maltose, mannitol, rhamnose, arabinose, xylose, trehalose and dulcitol but failed to attack lactose, sucrose or salicin in 30 days. Both strains of *S. morotai* rapidly produced acid and gas from inositol but *S. ball* failed to attack this substrate. They all produced acid in Jordan's tartrate medium in 24 hours at 37° C. and gave a pale lilac (±) reaction in Stern glycerol medium.

Antigenic Analysis.

S. morotai.

In slide tests with Salmonella O sera representing all the O factors up to and including XXXIX and also XLI strong agglutination occurred only with *S. kirkee* (XVII) serum. In tube tests, suspensions of *S. kirkee* and the two strains of *S. morotai* were all agglutinated by this serum to a dilution of 1/320. *S. kirkee* O serum absorbed with either strain of *S. morotai*

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failed to agglutinate the homologous organism at a dilution of 1/10. An O antiserum prepared against one of the strains of *S. morotai* and absorbed with the other strain or with *S. kirkee* also failed to agglutinate both strains of *S. morotai* at a dilution of 1/10. Before absorption the *morotai* O antiserum agglutinated *S. kirkee* and both strains of *S. morotai* to a dilution of 1/2,500. These results indicated that both strains of *S. morotai* had the same O factor which also occurred in *S. kirkee*. In the Arizona group of Edwards, West and Bruner (1947) a strain known as Sp 21 was described with the O-antigen XVII of *S. kirkee*. We therefore absorbed *S. morotai* O antiserum with Sp 21 and found that all agglutinins were removed for *S. morotai*. Also from Sp 21 O antiserum all agglutinins were removed by absorption with *S. morotai*. The diagnostic O factor for *S. morotai* was therefore designated as XVII.

Both strains of *S. morotai* were diphasic and H suspensions in phase 1 were tested against H antisera representing the factors of phase 1. Agglutination occurred only with antisera prepared against Salmonellas containing the factor *l*. Absorbed antisera were prepared according to the method of Edwards and Bruner (1940) for each of the factors *v*, *w*, z_{13}, z_{28} which may occur with *l*. Agglutination of both strains of *S. morotai* occurred with the serum for factor *v* only. Thus *S. morotai* contained factors *l*, *v* in phase 1. However, absorption tests indicated that phase 1 of *S. morotai* was not identical with that of *S. london*, *S. new-brunswick* or *S. potsdam*. Antiserum for phase 1 of *S. london* was absorbed with each strain of *S. morotai* in phase 1. Before absorption the serum agglutinated *S. london* and the two strains of *S. morotai* to a dilution of 1/6,000. After absorption *S. london* only was agglutinated to a dilution of 1/200. Antiserum prepared against phase 1 of one of our strains of *S. morotai* was absorbed with *S. london* phase 1. Before absorption the serum agglutinated *S. london* and our two strains of *S. morotai* to a dilution of 1/6,000. After absorption *S. london* was not agglutinated at 1/100 but both strains of *S. morotai* agglutinated in the serum to a dilution of 1/400. This titre was little reduced after a second absorption with *S. london* phase 1, which thus failed to remove all agglutinins for phase 1 of the homologous organism from *S. morotai* antiserum. Likewise *S. morotai* failed to remove all agglutinins for phase 1 of *S. london* from *london* antiserum. However, the level of remaining agglutinins was low. Furthermore *S. potsdam* phase 1 or *S. new-brunswick* phase 1 were agglutinated by *S. morotai* antiserum to the same dilution (1/6,000) as *S. morotai* but neither of these organisms exhausted the serum of agglutinins for the homologous organism. After absorption with *S. potsdam* or *S. new-brunswick* phase 1 the serum agglutinated both strains of *S. morotai* phase 1 to a dilution of 1/400.

Tests with *S. morotai* phase 1 antiserum and *S. dar-es-salaam* or *S. worthington* phase 1 (*l*, *w*) produced much the same results as those with *S. london*. In phase 1 *S. dar-es-salaam* and *S. worthington* were agglutinated by *S. morotai* antiserum to the same titre (1/6,000) as the homologous organism. After absorption with *S. worthington* or *S. dar-es-salaam* phase 1 the *morotai* antiserum still agglutinated both strains of *S. morotai* phase 1 to a dilution of 1/400. This reduction in titre was similar to that effected by *S. london*. To determine whether the residual titre after absorption with *S. london* represented a portion of the factor designated as *l* or a new factor typical of *S. morotai*, a mixed absorption was done with *S. morotai* antiserum and phase 1 of *S. london* and *S. uganda*. After absorption, the serum at a dilution of 1/100 failed to agglutinate phase 1 of the absorbing organisms or *S. morotai*. Therefore no individual new factor was present in *S. morotai*, which possessed a particularly comprehensive form of the factor *l*. Phase 1 of *S. morotai* may therefore be designated as *l*, *v*.

In phase 2 *S. morotai* agglutinated with antisera containing agglutinins for the factor 1 but not with antisera for factor *e*. Absorption of *S. newport* phase 2 antiserum with *S. morotai* phase 2 removed all agglutinins for the homologous organism. Prior to absorption the serum agglutinated phase 2 of *S. newport* and both strains of *S. morotai* to a titre of 1/6,000, but

after absorption no agglutination occurred at 1/100. *S. morotai* phase 2 antiserum agglutinated phase 2 of *S. newport* and *S. morotai* in a dilution of 1/6,000, but failed to agglutinate either organism at 1/100 after absorption with *S. newport* phase 2. Single factor antisera were prepared for phase 2 factors 2, 5, 6 and 7. Both strains of *S. morotai* agglutinated only with the serum for factor 2. Phase 2 of *S. morotai* was therefore represented as 1, 2.

The diagnostic formula of *S. morotai* is XVII; *l, v*; 1, 2.

S. ball.

This organism reacted strongly with O antisera produced against Salmonellas with the O factor IV. With absorbed antisera prepared for detection of the factors I or V, so often associated with IV, *S. ball* agglutinated only with the serum for factor I. O antiserum prepared against *S. typhi-murium* var. *copenhagen* was absorbed with *S. ball*, and O antiserum prepared against *S. ball* was absorbed with *S. typhi-murium* var. *copenhagen*. After absorption both antisera failed, at a dilution of 1/3, to agglutinate the homologous organism. The O factors of *S. ball* were therefore considered to be I, IV, XII.

S. ball was diphasic and in phase 1 agglutinated only with antisera prepared against Salmonellas containing the H factor *y*. *S. bareilly* phase 1 antiserum agglutinated *S. ball* phase 1 to the same titre (1/6,000) as the homologous organism. Absorption of this serum with *S. ball* rendered it incapable of agglutinating *S. bareilly* phase 1 at 1/100. After absorption with *S. bareilly* phase 1, *S. ball* phase 1 antiserum failed to agglutinate the homologous organism at 1/100. The phase 1 factor of *S. ball* was therefore designated as *y*.

In phase 2 *S. ball* agglutinated with all antisera prepared against Salmonellas containing the factor *e*. In tests with absorbed antisera representing the factors *x* and z_{15} which may be associated with *e* in phase 2, agglutination of *S. ball* occurred only with the *x* serum. As the *e, n, x* phase may differ somewhat among Salmonellas possessing it (Edwards and Bruner, 1941) further investigation of this phase of *S. ball* was undertaken. *S. abortus-equi* antiserum agglutinated *S. ball* phase 2 to the same titre (1/12,000) as *S. abortus-equi*. Absorption of this serum with *S. ball* phase 2 left no agglutinins for the homologous organism at 1/100. Antiserum was prepared against *S. ball* phase 2 and absorbed with *S. abortus-equi*. Before absorption the serum agglutinated *S. abortus-equi* and *S. ball* phase 2 to a dilution of 1/50,000. After absorption neither organism was agglutinated by the serum at 1/100. Phase 2 of *S. ball* was therefore considered to be identical with that of *S. abortus-equi* and was designated as *e, n, x*. *S. chester* phase 2 antiserum absorbed with *S. ball* still agglutinated the homologous organism at a dilution of 1/800 as compared with 1/25,000 before absorption. Similarly *S. ball* phase 2 antiserum absorbed with *S. chester* agglutinated *S. ball* phase 2 in a dilution of 1/2,000 as compared with 1/12,000 before absorption. To account for their observed differences among *e, n* phases Edwards and Bruner (1941) distinguished phase 2 of *S. chester* by the formula *e, n, x,* z_{17}, z_{19} and *S. abortus-equi* phase 2 by the formula *e, n, x,* z_{16}, z_{19}. From our results *S. ball* phase 2 should be represented by the formula *e, n, x,* z_{16}, z_{19}. Members of the z_{15} group, *S. glostrup* and *S. san-diego*, were both incapable of exhausting *S. ball* phase 2 antiserum of its agglutinins, but the residual titre was much the same as that found after absorption with *S. chester*. These results supported the previous evidence for the absence of z_{15} from *S. ball*.

The diagnostic formula of *S. ball* is therefore I, IV, XII; *y*; *e, n, x*. For more exact definition the phase 2 formula may be expanded to *e, n, x,* z_{16}, z_{19}.

Pathogenicity.

The two strains of *S. morotai* came from two typical cases of food-poisoning in a single outbreak in a small military unit on the island of Morotai. Ten cases occurred in all and *S. morotai* was the only pathogen isolated.

Unfortunately no details could be obtained concerning the gastro-enteritis case from which *S. ball* was isolated.

Both strains of *S. morotai* and the single strain of *S. ball* were injected intraperitoneally into mice in doses of 500×10^6 and 250×10^6 organisms. With the larger dose one out of four and two out of four mice inoculated with each strain of *S. morotai* died within two days, but none of the four mice receiving *S. ball* died within 14 days. With the smaller dose one out of four mice inoculated with one strain of *S. morotai* died in two days, three out of four inoculated with the other strain died in five days, and one out of four inoculated with *S. ball* died in one day. In all dead mice the injected organism was detected in the heart blood. Mice surviving for 14 days were sacrificed and from three of the nine remaining *S. morotai* mice and all the remaining *S. ball* mice the injected organism was recovered from liver and spleen.

DISCUSSION.

S. morotai is particularly interesting as it is the only other Salmonella so far recorded with the antigen XVII of *S. kirkee*. The occurrence of only one type, represented by only one or two strains, with a particular O antigen renders the position of that O component and its corresponding organism rather insecure in the Salmonella group, and *S. ballerup* whose antigenic factors, both H and O, have not recurred in any other recognized Salmonella has now been omitted from the group (Edwards, 1949). The position of *S. kirkee*, of which the original strain described by Bridges and Dunbar (1936) is the only example, has been maintained as it contains the well known Salmonella H factors *b*, 1, 2. Bornstein (1943) did not report *S. kirkee* for North America; Seligmann, Saphra and Wassermann (1943, 1946) did not find it among 3,000 human strains in America and Kauffmann (1941) and Wilson and Miles (1946) mentioned only the original strain. *S. minnesota*, which, like *S. kirkee*, is not accompanied in the diagnostic schema by any other Salmonella with the same O antigen, has been identified on various occasions from human and animal sources (Seligmann *et al.*, 1946; Edwards and Bruner, 1943; Hormaeche *et al.*, 1943) and is an established type. Its position is therefore different from that of *S. kirkee*. The discovery of *S. morotai* from typical human cases of gastro-enteritis supports *S. kirkee* as a member of the Salmonella group. The specific phase factors of *S. morotai* were not identical with those of *S. london* but no strong individual factor seemed to be present. We therefore attributed the observed differences to a variation in the components of the complex factor *l*, most components of which appeared to be present in *S. morotai*.

S. ball contained no new factors but illustrated again the already well-recognized complexity of the *e*, *n* phase. This phase in *S. ball* was identical with that of *S. abortus-equi* but differed considerably from that of *S. chester* which contained a strong factor of its own, different from any in *S. ball*, and presumably corresponding to z_{17} of Edwards and Bruner (1941).

Both our new types appear to be human pathogens producing typical gastro-enteritis. They did not seem to be highly pathogenic for mice.

SUMMARY.

Two new Salmonella types producing human gastro-enteritis are described.

The first type, for which the name *S. morotai* is suggested, has the diagnostic formula XVII; 1, *v*; 1, 2 and is represented by two strains from an outbreak of food-poisoning involving about 10 people on the island of Morotai.

The second type, for which the name of *S. ball* is suggested, has the diagnostic formula I, IV, XII; *y; e, n, x*. One strain was isolated from a human case of gastro-enteritis in South Australia. The *e, n, x* phase of this organism was identical with that of *S. abortus-equi* but different from that of *S. chester*.

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LOCAL RE-INNERVATION IN PARTIALLY DENERVATED MUSCLE: A HISTO-PHYSIOLOGICAL STUDY[1]

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In certain experiments on the cricothyroideus muscle of the rabbit, which is innervated by both the superior laryngeal and laryngeal plexus nerves, Exner (1885) found that after section of one of these nerves no histological evidence of muscle degeneration could be obtained, while complete degeneration followed section of both. Lederer and Lemburger (1907) showed by electrophysiological experiments that there was no overlapping innervation, making plausible Exner's supposition that the denervated muscle-fibres were re-innervated from the surviving nerve-fibres.

Weddell, Guttmann and Gutmann (1941) described decrease in anaesthetic areas of rabbit skin following cutaneous nerve lesion, even when regeneration of the damaged nerve could be excluded, and Speidel (1941), by day-to-day observation of individual nerve-fibres and endings, detected re-innervation of denervated regions of tadpole skin by nerve-fibres growing in from neighbouring regions.

Hines, Wehrmacher and Thompson (1945), van Harreveld (1945) and Weiss and Edds (1945) all described experiments designed to test electrophysiologically the possibility that, following partial denervation of muscle, the surviving nerve-fibres could establish new connections with the denervated muscle-fibres, before the severed nerve-fibres could regenerate. The first-named workers found that, after partially denervating hind-limb muscles of rats, the force of contraction elicited by stimulation of the surviving innervation increased after a period of a few days. The increase commenced on the third day, developed rapidly until the ninth day, and then gradually slowed down after the fourteenth day. The short period involved obviously excluded regeneration of the severed nerve stump. Similar observations were recorded in cats. van Harreveld, working on the femoral plexus in rabbits, obtained similar results extending over rather longer periods; however, regeneration of the severed nerve could still be eliminated. Histological examination indicated that very few muscle-fibres had atrophied, suggesting that local re-innervation of muscle-fibres had occurred. Weiss and Edds, working on the sciatic plexus of the rat, capped the severed nerve with methyl methacrylate resin, thus com-

[1] Since the completion of this work a preliminary note by Edds (Anat. Rec. (103) 534, 1949) has come to my notice, in which the re-innervation of partially denervated muscle by the branching of surviving nerve-fibres, is reported.

pletely preventing regeneration of the stump. Their histological findings indicated that neither atrophy nor hypertrophy of muscle-fibres had occurred to any significant extent. Hence the increase in the force-of-contraction of the muscle following partial denervation could only be accounted for by re-innervation of the denervated muscle-fibres.

Thus quite a large body of indirect evidence has accumulated, pointing to the existence of a mechanism by which denervated muscle-fibres can be re-innervated much more rapidly than by the normal process of axon-regeneration. The purpose of the present work is to investigate this phenomenon histologically, and to determine its character.

Experiments on the Partial Denervation of Muscle.

The Normal Appearance of Muscle Innervation in the Rat.

The normal picture of muscular innervation in mammals is constant, in contrast to that of non-mammals. The muscular nerve-branch ramifies within the muscle, breaking up into smaller branches until the "innervation node," or region of innervation, is reached when the branch breaks up into individual nerve-fibres which run short courses to end on muscle-fibres in characteristic motor end-plates. Each nerve-fibre lies in its own Henle sheath, which is made up of the endoneurial sheath which encloses it throughout its course down the nerve-trunk, and additional fibrous connective tissue which it only acquires when it leaves the terminal nerve-branch. A typical "innervation node" is shown in Fig. 2.

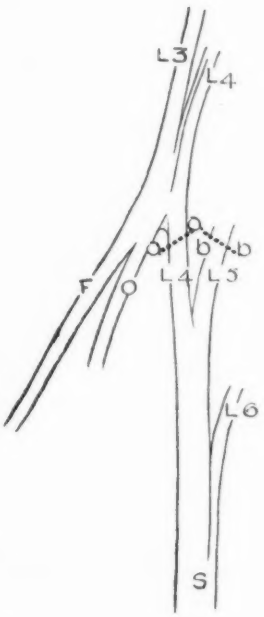

Fig. 1. Sciatic plexus of the rat. L3, 4, 5, 6—respective lumbar nerves. F = femoral nerve. O = obturator nerve. S = sciatic nerve.

a . . . . a level of section of L4.

b . . . . b level of section of L5.

There is little variation in the shape or size of the motor end-plate or nerve-fibre, and Cole (1947) has shown that the normal end-plate varies only from 20–30μ in greatest dimension. Further data regarding extremes in shape and size of end-plates are available in Carey's (1941) paper. There are no endings in mammalian muscle comparable to the "en grappe" endings found in Amphibians and Reptiles; all nerve-fibres are medullated, and the nerve-fibre seldom branches once it has left the terminal nerve-branch. Wilkinson (1929) has already commented on the absence of ultraterminal end-plates, non-medullated collaterals and "en grappe" endings in mammals, while Mather and Hines (1934) have contrasted the constancy of intramuscular innervation in mammals with the extreme variability found in the lower vertebrates.

The courses taken by nerve-fibres in their paths along the nerve-trunk are such that fibres originating close together in the plexus separate, and intertwine with others lower down the nerve-trunk. In the rat all segments participating in the sciatic plexus contribute to the innervation of each of the muscles used in the experiments described below. As a result, when

a single segmental nerve is severed, normal and degenerating nerve-fibres are intertwined lower down the trunk and within the muscle. Since most of the evidence supports the contention that individual mammalian muscle-fibres possess only one end-plate each—Denny-Brown and Pennybacker (1938), and Fort (1940)—it follows that partial denervation of a nervous plexus results in an admixture—even within the fascicles—of denervated and innervated muscle-fibres.

Experimental.

In sixteen rats the sciatic plexus was partially denervated under nembutal anaesthesia (45 mg./kg. body-weight) using substantially the surgical approach of Weiss and Edds. The plexus (see Fig. 1) is made up of L4, L5 and L6, contributing in varying proportion. In a few cases L6 does not contribute at all. The femoral nerve is exposed as soon as the iliacus muscle is split, and can be traced to its connection with L4, thus exposing the sciatic plexus. L4 or L5 was sectioned and resected for a few millimetres to facilitate *post-mortem* confirmation. The animals were killed from one to seventy-seven days after operation, and the plexus examined to confirm denervation. Gastrocnemius, soleus and tibialis anterior muscles were prepared histologically, mostly by the Loewit gold-chloride-formic acid technique, teased and mounted in glycerin jelly, although a few preparations were made by the Rogers (1931) silver-on-the-slide method. The former method has the advantage of permitting nerve-fibres to be followed for comparatively great distances. Muscles from the unoperated side, and from normal animals, served as controls. Possible complications from regeneration of the severed nerve-stump were in all cases eliminated, for after fourteen days only a transparent bridge crossed the gap in the nerve, and only after twenty-one days did axons pass over it.

On the first and second days after partial denervation, the only changes observable in the prepared material are those of Wallerian degeneration in the severed axons; however on the third day there appear, scattered amongst the normal and degenerated nerve-fibres, a few fine and thread-like axons, measuring 0·5μ or less in diameter. They contrast with normal axons in their lack of nodes of Ranvier, and their small diameter. The courses they take are similar to those of normal nerve-fibres, and the majority form end-plates on muscle-fibres, although a few end blindly in swollen tips some distance from muscle-fibres. The end-plates formed by these fine axons are diminutive, and their arborisations are extremely delicate (see Figs. 3 and 4).

The fine fibres are enclosed in an endoneurial (Henle) sheath, which is usually of the same diameter as that investing a mature nerve-fibre. In a few cases a fine axon is seen lying within an endoneurial sheath which extends beyond it in the direction of a muscle-fibre, and the axon terminates in a swollen tip, resembling the "growth cones" described by Speidel and Weddell *et al.* In very few cases a degenerated end-plate can be seen alongside a smaller immature one, prompting the suggestion that in the majority of cases the old sole-plate is re-innervated.

On the fourth day far more fine axons may be seen, and can be traced back to their origin as fine branches of normal axons (Fig. 4). Most of these arise 100–200μ from the end-plate as branches of nerve-fibres within the terminal nerve-branch, some as branches of solitary nerve-fibres. In some cases two fine axons arise at a single node (Fig. 5) and many branches may arise from a short length of a single axon.

By the sixth day the fine fibres outnumber the mature ones in some regions, always running together with normal and degenerated nerve-fibres. In the otherwise empty endoneurial sheaths of degenerated nerve-fibres characteristically enlarged neurolemmal cells with prominent granular nuclei may be seen, constituting the "cords of Büngner," and they are also apparent within endoneurial sheaths along which the new fibres have grown, being readily distinguishable from normal neurolemmal cells by their large size. By the eighth day the fine fibres are beginning to thicken, undergoing the characteristic "maturation" process described

by Young and co-workers, the thickening commencing at the proximal end and progressing distally. The further history of growth is largely a process of maturation, so that, after seventy-seven days, the picture appears comparatively normal. Nodes can now be seen, suggesting that myelination is proceeding, although the myelin sheath itself is not visible in gold-stained material. Speidel has already stressed the rôle of myelination in maturation.

Other processes occur side by side with this outgrowth of fine nerve-branches, characterized by the same general trend of activation to outgrowth, but not always resulting in re-innervation. Many end-plates enlarge, reaching out across and along the muscle-fibre until, in some cases, a length of 100μ or more is reached (Fig. 7). Processes grow out from other end-plates, sometimes passing out through the sarcolemma and penetrating neighbouring muscle-fibres, forming ultra-terminal end-plates. In several instances a denervated muscle-fibre is re-innervated both by a fine axon, and by an ultraterminal outgrowth from a neighbouring end-plate (Fig. 6). In a few cases a branch grows out from an axon very close to the end-plate. This process does not possess an endoneurial sheath, and never grows for any great distance; its tip is swollen and resembles a growth cone.

Interpretation.

The possession of an endoneurial sheath resembling that occupied by a mature nerve-fibre, as early as three days after operation, suggests that the growing axon has invaded an empty sheath originally occupied by a now degenerated nerve-fibre. The presence of enlarged neurolemmal cells within this sheath further supports this hypothesis, as does also the absence of intermediate stages in the laying down of new sheaths. The presence of a growing nerve-fibre capped by a growth-cone, within an endoneurial sheath which extends beyond it, seems further to support the hypothesis that the outgrowing axon-branch encounters an empty endoneurial sheath, penetrates it and grows down it to re-invade the old sole-plate and form a new end-plate. This would account for the rarity of degenerated end-plates. Gutmann and Young (1944) have shown that, when muscle is re-innervated relatively soon after denervation, the majority of the axons run down the old endoneurial sheaths to re-innervate the original sole-plates. Weddell *et al.* (1941) have shown that the nerve-fibres re-innervating denervated areas of rabbit skin from neighbouring cutaneous plexuses invade the sheaths of degenerated nerve-fibres.

The origin of most of the new innervation by outgrowth of branches from intact axons is similar to the processes described by Weddell *et al.*, and also to those phenomena described by Speidel in the skin of the tadpole tail, suggesting that the adult nerve-fibre retains some of the lability of the embryonic tissue. Whether only the region of an existing node is capable of sprouting or whether outgrowth is preceded by new node formation cannot be determined without direct observation, but Speidel has again thrown light on the question when he pictures mitosis of a neurolemmal cell, followed by node formation between the daughter cells, and then outgrowth of a branch from the node. No trace has been found of the blindly-ending "free terminals" mentioned by Weiss and Edds as a possible source of re-innervating branches, and it seems clear that no such structures play any part in the process of local re-innervation.

Two mechanisms have been suggested by which branching of surviving nerve-fibres could result in re-innervation of denervated muscle-fibres. van Harreveld (1945) suggested that, following partial denervation of the muscle, nerve-fibres in the muscle might be ruptured, either by contraction of some of the muscle-fibres in the fascicles, or by the partially paralysed limb adopting abnormal positions of rest. The damaged nerve-fibres, during regeneration, might then branch and re-innervate the previously denervated muscle-fibres. Weiss and Edds (1945) suggested that, before the surviving nerve-fibre could branch to re-innervate denervated muscle-fibres, it must withdraw its connections with its end-organ. The analogy here is obviously with the regeneration of a severed nerve-fibre, since in regeneration nerve-fibres often give off many

fine branches (Cajal, 1928). The actual process, as shown above, is however clearly different from those suggested by the above authors, and closely resembles those described by Speidel.

Since the growing axons have a distance of only 100–200μ to traverse in order to reach the sole-plate and form new end-plates, and since the guiding mechanism is similar to that available to a nerve-fibre growing down a nerve trunk, the entire process of growth may involve only one or several hours. The delay of three days between section of the nerve and the commencement of outgrowth of branches probably represents the time involved in the development of a stimulus to re-innervation. The times of first appearance of nerve-branches, and onset of maturation, correspond closely with those obtained physiologically by Hines *et al.*, suggesting that the fine axons become conducting soon after they reach the sole-plates. This is in agreement with the findings of Gutmann and Young (1944).

The phenomenon of local re-innervation has been inferred from physiological experiments in the muscles of the rat (Hines *et al.*, Weiss and Edds), rabbit (van Harreveld), cat (Hines *et al.*) and dog (Frederick and Kosman, 1948).

Mechanism of Regeneration Following Partial Denervation.

van Harreveld (1947) investigated the nature of the stimulus to re-innervation and found that the re-innervation could be accelerated if muscles near to the partially denervated one were completely denervated. This seemed to indicate that some accelerator substance diffused out of the totally denervated muscle and into the partially denervated one. He then injected ether extracts of fourteen day denervated muscle into a partially denervated one, and claimed that re-innervation was again accelerated. Ether extract of normal muscle did not have this effect. This was taken to indicate that a slightly diffusable substance was released in the muscle after denervation, and accelerated re-innervation.

The substance postulated by van Harreveld could be released either from the muscle-fibre undergoing degeneration, or from the disintegrating nerve-fibre or end-plate. This second possibility was favoured by Weiss (1945) who suggested that some substance, diffusing out of the degenerating nerve-fibre or end-plate could serve as an irritant to intact nerve-fibres nearby, and evoke outgrowth. Weddell *et al.* suggested that the contents of the sheath of the degenerating nerve-fibre might serve as the stimulus to re-innervation of denervated skin. The relatively early onset of re-innervation suggests that the stimulus for outgrowth arises from degenerating nerve, rather than from denervated muscle-fibres, as the period of delay is comparable with times of degenerative change in nerve, e.g. loss of conductivity in mammalian nerve, as obtained by Gutmann and Holubar (1949).

The possibility that sprouting of nerve-fibres is prevented in normal muscle by the presence of an inhibitor, which would disappear after denervation, is discounted by the work of Aitken (1949). He found that the growth of nerve into denervated muscle, and the subsequent re-innervation, could not be prevented by the injection of extract of normal muscle.

Experimental.

The following experiments were designed to test whether an evocator is released in denervated muscle which can evoke outgrowth from the nerve-fibres in normal muscle, and to trace its origin.

(a) *Effects of Injection of Extracts of Denervated Muscle and Degenerated Nerve.*

The sciatic nerves in a number of rats were severed on the right side, and allowed to degenerate for seven days. The entire musculature below the knee was then homogenized in about 2 ml. of 0·85 p.c. saline, while the sciatic nerve from below the lesion was separately homogenized in about 1 ml. of saline. The extracts were injected into the muscles of rats,

DESCRIPTIONS OF ILLUSTRATIONS.

All photomicrographs are from gold-stained, teased, muscles of the rat.

Fig. 2. A "node of innervation" in normal rat muscle. Note the absence of branching in axons once they have left the terminal nerve-branch. (× 138).

Fig. 3. A newly-formed, fine non-medullated axon running alongside the mature one. The endoneurial sheaths of both are visible at some points. From a rat in which L4 was severed eight days previously. (× 375).

Fig. 4. An immature axon running beside mature ones, showing its origin as a branch of a mature axon. Rat, eight days after section of L4. (× 375).

Fig. 5. A mature axon from which originate three fine, non-medullated branches. In the upper portion of the nerve-fibre two of these branches are seen to arise at a single node. The end-plate of the fibre on the right is only visible as a ghost, but is shown separately above, the double arrow indicating ghost and inset images. Note the delicate character of the end-plate arborisations. Rat, seven days after section of L4. (× 375).

Fig. 6. Two muscle-fibres *a* and *b* lie side by side, the lower, *b*, being innervated by a mature axon, the upper, *a*, by a fine immature axon with a diminutive end-plate. From the end-plate on muscle-fibre *b* an ultraterminal process has grown out and innervated muscle-fibre *a*. Rat, ten days after section of L5. (× 375).

Fig. 7. A greatly enlarged end-plate, 100μ in length, is seen in the upper left corner. The enlargement of end-plates occurs in partially denervated muscles, and probably represents a variation in response to neurocletin. Rat, six days after section of L4. (× 375).

Fig. 8. A process, tipped by a swollen "growth cone," has grown out from an end-plate and passed down the muscle-fibre under the sarcolemma. From tibialis anterior of rat, injected daily with homogenate of seven day degenerated sciatic nerve in saline, and killed after four days. (× 375).

Fig. 9. A fine nerve-fibril growing out from an end-plate, passing through the sarcolemma, ends blindly. The tip is invisible, lying outside the plane of focus. From tibialis anterior of rat, six days after injection of ether extract of ox spinal cord emulsified in propylene glycol. (× 375).

Fig. 10. A fine fibril has grown out from an end-plate, passing across the muscle-fibre, still under the sarcolemma. Ten days after injection of a saline homogenate of seven day degenerated rat sciatic nerve. (× 375).

Fig. 11. A long outgrowth from an end-plate has passed out through the sarcolemma and is growing parallel to the muscle-fibre and below it, terminating in a swollen growth cone. The process is about 120μ long. Rat, injected with a saline homogenate of rabbit brain, killed forty-eight hours later. (× 280).

Fig. 12. An outgrowth from an end-plate has passed out from the muscle-fibre and ends blindly near another muscle-fibre. Rat, six days after injection of a saline homogenate of seven day denervated rat muscle. (× 375).

Fig. 13. An ultraterminal outgrowth from the middle end-plate grows down and fuses with the end-plate on the right. Rat, three days after injection of ether extract of egg-yolk, emulsified in propylene glycol. (× 375).

Fig. 13a. This is a photograph of the middle end-plate from Fig. 13 taken at a slightly higher level, to show the outgrowth of a fine process from the nerve-fibre just before it enters the end-plate, passing down over the end-plate and forming two branches which end blindly. The origin of this fine branch is indicated by the arrow. (× 375).

Fig. 13b. This is a photograph of the end-plate on the right in Fig. 13 to show the outgrowth of another process from it on the right side. This process appears to be a continuation of the process which fuses with the arborisations of this end-plate.

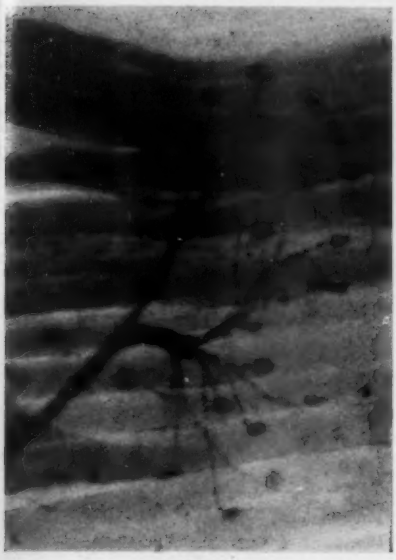

Fig. 2

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

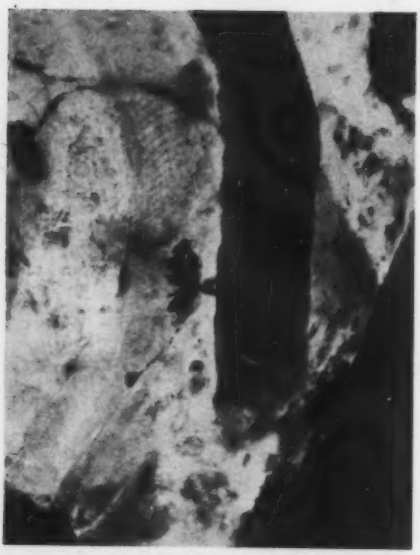

Fig. 8.

Fig. 9.

Fig. 10.

Fig. 11.

Fig. 13.

Fig. 13a.

Fig. 13b.

Fig. 12.

usually tibialis anterior, the volume per injection being kept below 0·5 ml. The extracts were injected daily, as it was feared that any evocator substance present might be absorbed too rapidly to maintain an effective concentration. The animals were killed 5–7 days after first injection, and the muscles prepared by the gold chloride method.

On examination of the stained material, a few end-plates, about 0·5 p.c., showed ultraterminal outgrowths, both in the case of nerve extract and muscle extract. Some of these outgrowths consisted of fine fibrils running short distances along the muscle-fibre under the sarcolemma, and terminating in swollen tips, while others passed out through the sarcolemma and wandered in the perimysial spaces (Figs. 8, 10, 12). No sign of new end-plate formation was ever seen, and the tips of the fibrils were usually swollen, resembling growth cones, and suggesting that growth was proceeding at the time of preparation of the muscle. Control injections of homogenate of heart and liver, and saline alone, produced no such effects, although it was found that if a volume greater than 1 ml. of any of these fluids was injected, small spherical, dark-staining bodies were released from the end-plate and passed into the sarcoplasm. These are thought to be comparable with the "neurosomes" of Carey (1948).

Centrifugation of the homogenate at 2,000 r.p.m. for ten minutes did not remove the evocator activity of the supernatant fluid, while the residue, resuspended in saline and centrifuged again, had no evocator activity. Denervated muscle from rabbits was homogenized in saline and found to be equally effective when injected into rats, and a single injection of homogenate was found to be adequate.

That the stimulus is chemical seems to be quite clear, for regeneration of nerve-fibres damaged while performing the injection is eliminated by control injections of the ineffective tissue extracts, and by the complete absence of degenerating nerve-fibres from the stained material. It is proposed to call the process of evocation "neuroclesis" (from "neuron" a nerve, "clesis" a call) and the evocator a "neurocletin."

Although most of the outgrowths were ultraterminal in origin, occasional branches arose from the nerve-fibres themselves, close to the end-plate. These grew for varying distances, ending in swollen growth cones. They resemble the branches of nerve-fibres, observed in partially denervated muscle, which arise too far down the nerve-fibre to encounter an empty endoneurial sheath.

Since both degenerating nerve and denervated muscle contain this neurocletin, its origin from nervous tissue is indicated. The following series of experiments was carried out to determine whether the presence of degenerating nerve was the essential factor for neuroclesis.

(b) *Neurocletic Effects of Neurografts.*

In five rats sciatic nerve homografts were threaded through the eye of a surgical needle and passed down through the body of the gastrocnemius muscle, the nerve being left implanted there. The animals were killed seven to ten days later and the muscles examined for persistence of the grafts, then prepared histologically.

The nerve-graft could be expected either to produce a homograft foreign-body reaction, in which case it would be rapidly absorbed by phagocytosis, or to survive and degenerate *in situ*. Both histologically—Nageotte (1910) and Cajal (1928)—and chemically—Johnson, McNabb and Rossiter (1949)—the degeneration of nerve *in vivo* is essentially similar to that occurring *in vitro*, so that a persistent graft should behave similarly to the degenerating nerve in partially denervated muscle. In the first animal, examined after seven days, no trace of the inserted nerve remained; within the muscle nerve-fibres and end-plates showed no changes from the normal. In the four following cases the grafts still persisted seven to ten days after insertion and the ends could be seen protruding out from the muscle surface. In all of these cases occasional end-plates showed ultraterminal outgrowths, while a few nerve-fibres showed

fine, blindly-ending, branches, originating close to the end-plate. In the first experiment, it may be assumed that the rapid absorption of the graft prevented the neurocletic stimulus.

The reaction of the muscular innervation after ten days' exposure to degenerating nerve was rather less than that obtained after shorter periods of injection of extracts and this may be attributed to the different distribution of neurocletin in relation to the responsive tissues.

(c) *Neurocletic Effects of Extracts of Normal Tissues.*

Since the neurocletic substance could be either present in the normal nerve-fibre and released from it during degeneration, or be formed during the chemical changes following degeneration, homogenates of normal nerve and muscle were prepared and injected into rat muscles. The results obtained from these were similar to those obtained from degenerated tissue extracts, suggesting that neurocletin is a constituent of normal nervous tissue. Homogenates of rabbit spinal cord and brain were next prepared and injected, and the animals killed after two days. The prepared material yielded ample evidence of neurocletic response; rather more numerous outgrowths from end-plates were obtained, some reaching 100μ or more in length (Fig. 11). The response obtained was greater than to any previous extract, and the short period allowed here further suggests that the delay in the appearance of new nerve-branches in partially denervated muscle is largely due to the slow release of neurocletin. The greater response can be correlated with the higher concentration of nervous tissue in the homogenate, i.e. higher concentration of neurocletin.

A substance, which can be extracted from normal peripheral nerve and white matter of spinal cord and brain, and which serves to accelerate the regeneration of severed sensory nerve trunks to the cornea in rabbits, has been described by Koechlin and von Muralt (1947) and called "N.R." (neuro-regeneratory substance). It is water soluble, passes through a dialysing membrane, is readily soluble in 90 p.c. alcohol, insoluble in ether, and moderately thermostable. To determine the relationship of neurocletin to the N.R. substance, three extracts were prepared from ox spinal cords. The cords were stripped of the dura- and pia-mater and homogenized in the various solvents as follows.

(a) The spinal cord was homogenized in sufficient distilled water to make a viscous liquid, then placed in a cellulose bag porous to molecules as large as M.W. 10,000, with an equal volume of distilled water outside the bag. After twenty-four hours the outer solution was removed and concentrated to one quarter of the original volume under reduced pressure at 30°C.

(b) Two hundred gm. of cord were homogenized in about five hundred ml. of anaesthetic ether, agitated, and after twenty-four hours filtered, and the filtrate evaporated under reduced pressure at room temperature. The residue was a yellow waxy material.

(c) A homogenate of spinal cord in 90 p.c. alcohol was prepared as for the ether extraction, and similarly evaporated, yielding a white fatty material. The dialysate was injected directly into the rat muscle, the other extracts were homogenized in maize oil to form emulsions, which were then injected.

The dialysate had not the slightest effect on the nerve-fibres or end-plates, although injected daily for three days. The alcoholic extract had a slight effect, producing a few short outgrowths, while the ether extract produced a much greater response. After four days, many long outgrowths were to be seen, wandering in the perimysial spaces. Further animals were injected with ether extract of ox spinal cords emulsified in propylene glycol, and the responses were consistently great; in regions of the muscle up to 30 p.c. of the end-plates developed ultraterminal outgrowths. Injections of maize oil and propylene glycol did not evoke any response, nor did an emulsion of vaseline in propylene glycol, selected for its similarity to the ether extract of ox cord in mechanical properties. Ether extract of ox cord produced inflammatory reaction in the muscle into which it was injected.

Ether extracts of denervated and normal rabbit muscle were next prepared. The spinal cords of rabbits were exposed by drilling through the third lumbar vertebra, and severed at the level, while the cauda equina below was pithed. This resulted in complete denervation of both hind limbs. After three days the animals were killed and the muscles stripped and minced, yielding about 200 gm. of muscle. This was agitated with ether, and after twenty-four hours filtered and evaporated. Normal muscle was similarly extracted. The extracts consisted of yellowish fat in young rabbits, whilst older rabbits yielded a viscous oil, probably due to the presence of unsaturated fat in the intramuscular spaces. These extracts were emulsified in propylene glycol and injected into rat muscles. Both extracts produced blindly-ending outgrowths from nerve-fibres and end-plates, some travelling long distances in the perimysium. If the substance extracted by van Harreveld, which accelerated re-innervation after partial denervation, is identical with neurocletin, these results are contrary to his, since he claimed that ether extract of normal muscle was inactive.

Finally, ether extract of egg-yolk was prepared and homogenized in propylene glycol. Injection of this extract produced the greatest response of all; in some regions of the muscle up to 50 p.c. of the end-plates and many of the nerve-fibres produced long, blindly-ending outgrowths which twined around other nerve-fibres, approached end-plates, sometimes turning away, sometimes even fusing with the arborisations of other end-plates (Figs. 13, 13a, 13b). This activity of egg-yolk lipoids is not entirely unexpected as many growth-promoting substances have been extracted from this source.

The relationship of neurocletin to N.R. substance is not clear, since the dialysate, which should be rich in N.R., was completely inactive. The ether extract which, according to Koechlin and von Muralt, should be completely devoid of N.R., was the most active neurocletic extract. N.R. is a readily diffusible blood-borne substance, while neurocletin is only slightly diffusible. It seems significant that several different substances, or groups of substances, behave either as evocators or accelerators to nervous growth; whether N.R. will behave as a neurocletin is uncertain since it may be rapidly absorbed, and therefore not remain in one site long enough to exert any local effect. The different substances may differ in molecular structure only by the possession of polar groups which vary their solubilities and hence their site of action.

Some evidence of the localization of the neurocletic substance within the nerve-fibre was sought, by means of extracts of grey and white matter of the ox brain. The brain was stripped of pia-mater and the cerebral convolutions opened out and scraped clean of grey matter. White matter was exposed, but any carried over with the grey matter was teased free and discarded. Equal masses of grey matter obtained by this means, and white matter from the medulla, were extracted with equal volumes of ether, the evaporated extracts being then emulsified in equal volumes of propylene glycol. Rats were injected with 0·5 ml. of either emulsion. White matter extract produced a typical neurocletic response, grey matter extract was ineffective. Since the essential difference between the nerve-fibres of the two regions of the brain is the presence of the myelin sheath in the white matter, it is in this myelin sheath that the neurocletic substance may now be localized.

DISCUSSION.

The region most frequently responding to the injected neurocletin is the end-plate, responsiveness apparently decreasing in a disto-proximal gradient along the nerve-fibre, resembling the cyanide-susceptibility and other gradients along the amoeba pseudopod. Carey (1941) has indicated that the end-plate retains some of the amoeboid properties described by Harrison for the tip of the growing axon.

Weiss (1945) has pointed out that unconnected nerve-fibres can persist in muscles without resorption, indicating that the adult tissue has lost some of the lability of the embryonic stages, since Speidel (1942) has shown that collateral nerve-branches may be readily resorbed in the tadpole.

The phenomena characterizing re-innervation following partial denervation may be analysed into three phases. The first is the evocatory "neurocletic" process in which the diffusion of neurocletin from the myelin sheath of the degenerating nerve-fibre stimulates the nearby surviving axon to sprout a branch, or in a few cases, the end-plate, to give off an ultraterminal process. This response can be duplicated by artificially administered neurocletin. The second phase is the orientation of the growing axon, in which it is guided towards the muscle-fibre which it can innervate. The growing tip of the new axon approaches the empty endoneurial sheath from which is diffusing the neurocletic substance, penetrates and passes down the sheath toward the old sole-plate. This efficient guiding mechanism accounts for the rapidity of the re-innervation process. Artificially induced outgrowths cannot utilize any such guiding mechanism, and follow random courses. The ability of growing nerve-fibres to fuse with ramifications of end-plates seems surprising, but this has been observed in several cases. Figs. 13, 13a, 13b illustrate this phenomenon. Gutmann and Young (1944) observed this random orientation of nerve-fibres re-innervating muscles after long periods of denervation, when the endoneurial sheaths have become fibrosed, and are no longer penetrable. The nature of the orientating structures assisting growing nerve-fibres has been discussed by Weiss (1934) who stressed the importance of ultrastructural patterns in the ground substance of connective tissues.

The third phase in the re-innervation process is the formation of new end-plates. This process is apparently rapid, being assisted by the brief duration of denervation, since the sole-plate can have undergone little atrophy. In artificially induced neuroclesis new end-plates are never observed, their formation being presumably conditioned by the presence of denervated muscle-fibres. Thus the innervated muscle-fibre is apparently resistant to penetration by growing nerve-fibres except in rare cases (Fig. 6). Fort (1940) has discussed the conditions governing re-innervation of muscle-fibres, and suggests that several days after denervation the muscle-fibre becomes receptive to the nerve-fibre and may then be re-innervated. It is possible that the muscle-fibre, soon after innervation, undergoes some membrane-penetrability change, comparable with that occurring in the monospermic ovum, in which the cell-membrane becomes impenetrable to further sperm after one has passed through. In the few cases in which double innervation has been observed (Fig. 6) the second nerve-fibre may have entered the muscle-fibre before the membrane-penetrability change was complete.

Certain observations of Weiss and Edds (1945) inclined them to the view that sprouting of surviving axons occurred also in the nerve-trunks, since they

observed fine, non-medullated axons in the sciatic nerve distal to the lesion. They considered that these could not have originated from the severed fibres, since these had been "capped." However, Edds (1949) has further investigated this phenomenon and concluded that these fibres have regenerated from the severed nerves by way of imperfections in the cap. This evidence further supports the view that a response gradient exists, responsiveness being greatest at the motor end-plate and decreasing proximally.

The evidence regarding re-innervation following partial denervation, and the fact that it does not always occur at the old sole-plate, throws new light on the question of plurisegmental innervation of mammalian muscles. Agduhr (1919, 1939) asserted that individual muscle fibres in certain muscles of mammals possessed several end-plates derived from nerve-fibres from different spinal segments, and that these end-plates often lay close together. This has been denied by many workers—Fort (1940), Denny-Brown and Pennybacker (1938), Wilkinson (1931)—and his experimental evidence now seems capable of a different interpretation. After partially destroying nervous plexuses, he was able to show, a few days later, a normal and a degenerating end-plate side by side on the same muscle fibre. He claimed that the degenerating end-plate represented the innervation from one spinal segment, the normal one that of the other segment. However, it is possible that the normal end-plates observed on these fibres were recently formed by the process described above, and that, in the material he was working with, re-innervation in sites other than the old sole-plate is common.

This lability of the adult nervous tissue could be of practical value in the treatment of partial muscular pareses such as that following poliomyelitis. Billig, van Harreveld and Wiersma (1946) attempted to utilize this phenomenon by means of "neurotripsy." This involved the crushing of the nerve close to the muscle, or the forceful massage of the muscle with a polished brass rod. The ruptured nerve-fibres were expected to form more branches when they regenerated, and re-innervate more muscle-fibres. Their clinical results were not very significant, and Frederick and Kosman (1948), repeating their experiments on partially denervated dog muscles, found the treatment to be ineffectual. Gutmann and Young (1944) have shown that the presence of an intact system of endoneurial tubes is the most important requirement for rapid and complete re-innervation, so that rupture of both axons and endoneurial sheaths following neurotripsy must certainly complicate the mechanism of regeneration.

Neurocletin could serve as a basis for treatment, designed to promote re-innervation of partially denervated muscle when such re-innervation does not occur spontaneously. If human nerve has not lost the competence to respond to neurocletin, re-invasion of empty endoneurial sheaths might be accomplished, resulting in improvement of muscle function.

SUMMARY.

It has been found that the re-innervation of muscle following partial denervation commences on the third day following operation, by means of branches growing out from surviving nerve-fibres and end-plates. The "ultraterminal" outgrowths innervate neighbouring muscle-fibres, while the axon-branches invade the empty endoneurial sheaths of degenerated nerve-fibres. The stimulus is nervous in origin, being brought about by the diffusion of an ether-soluble substance out of the myelin-sheath of the degenerating nerve-fibre. Re-innervation is rapid, and usually occurs at the site of the old sole-plate.

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STUDIES ON STRAINS OF *CORYNEBACTERIUM EQUI* ISOLATED FROM PIGS

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Corynebacterium equi has been described as the cause of various pathological conditions in many different animals. It has been isolated from pulmonary abscesses in foals (Magnusson, 1923; Bull, 1924; Lind, 1939; Bruner, Dimock and Edwards, 1939; Resketnyak, 1940; Flatla, 1942); from purulent lesions in the lungs of calves (Holtman, 1945); from the genital tract of barren mares and from aborted equine foetuses (Bruner, Dimock and Edwards, 1939). It has been recovered from the kidney of a horse suffering from acute nephritis (Carne, 1927); from cases of bovine sterility (Craig and Davies, 1940); and from a buffalo-cow and a mare following abortion (Rajagopalan and Gopalakrishnan, 1938; Mitscherlich, 1942). As well *C. equi* frequently infects pigs, and reports have come from the Scandinavian countries (Holth and Amundsen, 1936; Magnusson, 1940; Holth and Prag, 1941; from Denmark (Bendixon and Jepsen, 1938); Jespersen, 1938, a and b; Plum, 1938, 1939, 1940, a and b); from France (Verge and Senthille, 1942); from Great Britain (Cotchin, 1943); from America (Feldman, Moses and Karlson, 1940); and from Australia (Collins, 1939; McDonald, 1942); that this organism has been isolated from tuberculous-like lesions in the lymph glands of the head and neck of these animals.

The present work is an extension of the earlier work, and in it an attempt is made to look for *C. equi*-like organisms in lesions resembling tuberculosis in the submaxillary lymph glands of pigs slaughtered in South Australia; to identify them as strains of *C. equi* after comparing their morphological, cultural, biochemical and serological reactions with those given by the classical strain, *C. equi Magnusson;* and, lastly, to gather some information on the incidence of the organism in the diseased glands.

Materials and Methods.

Source and Isolation of the Strains of C. equi.

Included in the study were 21 cultures of *C. equi* and 7 cultures of diphtheroids which were unrelated to *C. equi* but were used mainly for comparison in the serological work. Five of the strains of *C. equi* were obtained from the Division of Animal Health and Nutrition, C.S.I.R.O., Melbourne. The cultures were as follows: *C. equi* Magnusson; *C. equi* Holth; *C. equi* N.C.T.C. 1621; *C. equi* Carne S. N. 1321—from the kidney of an 8 year old mare; *C. equi* Carne S. N. 4563—from the lung of a foal.

The remaining 16 strains of *C. equi*—subsequently labelled "C," plus an appropriate number—were isolated locally from the submaxillary lymph glands of pigs that showed tuberculous-like lesions in no other part of the body. The specimens were collected in 50 p.c. alcohol (McDonald, 1942) and were forwarded to the laboratory. Here they were aseptically removed, sectioned and examined macroscopically for the presence of the typical small lesions containing whitish material which was readily expressed, like tooth paste, on touching with a sterilized wire, leaving a shallow depression with a slightly raised edge in the gland tissue. Representative portions of the specimens were transferred to a mortar, and were ground with sterile sand. Subcultures were made into nutrient broth. These tubes were incubated at 37° C. for 24 hours and were then examined for the presence of *C. equi*. The remainder of the material was treated with 2N HCl for 15–20 minutes, and neutralized with an equivalent amount of 2N NaOH. After centrifugation, the supernatant was removed and the deposit smeared onto glycerine-egg and egg and saline media. These inoculated tubes were incubated at 37° C. and were examined macroscopically and microscopically, at regular intervals, for the presence of tubercle bacilli. If no growth occurred the tubes were discarded after 12 weeks of incubation. The cultural procedures employed were intended (1) to detect the presence of *C. equi*, and (2) to demonstrate the presence of viable tubercle bacilli.

Serological Work.

Antisera were prepared against the Magnusson, Holth, Carne SN4563 and Carne SN 1321 strains of *C. equi* and against three locally isolated strains—C63, C65 and C113—by injecting rabbits intravenously with large doses of formalin-killed organisms from 24 hours agar-slope cultures. Satisfactory antisera were obtained by giving the rabbits 9 injections (increasing in amounts of from 1 ml. to 9 ml.), the first four at one day intervals, the last five at 2 day intervals. The rabbits were bled on the 10th day after the last injection, the serum was collected and preserved with 0·25 p.c. phenol.

The acid extracts of *C. equi* organisms used in the precipitation tests were prepared according to the method of Bruner, Dimock and Edwards (1939). The precipitation tests were carried out by the qualitative method—0·3 ml. of clear extract was layered onto an equal amount of undiluted antiserum. Antigen and serum controls were included and readings were made at room temperature after 3–5 minutes. A well-defined white ring developing at the interface of the two reagents was recorded as a positive test.

Agglutination Tests.

Slide and tube agglutination tests were performed. For the slide tests a thick suspension of organisms from a 24 hour, agar culture was prepared in normal saline. One loopful of this live suspension was thoroughly mixed, with one loopful of undiluted antiserum. The slide was rocked gently and the test read for agglutination after 3–5 minutes.

The suspensions used in the tube agglutination tests consisted of heat-killed organisms, and were prepared by washing off the growth from 24 hour, agar cultures in saline, and heating the concentrated suspension at 56° C. for ½ hour. Before use these suspensions were standardized to an opacity equal to that of 1,000 million *Bact. coli* per ml. The sera were used at dilutions of 1 in 2·5 to 1 in 320. Antigen controls were included and the tests were read after 18 hours' incubation at 55° C.

Complement Fixation Tests.

The preparations of *C. equi* used as antigens in the complement fixation tests consisted of the residue of organisms left after extraction with acid. They were prepared according to the

method of Bruner, Dimock and Edwards (1939). Before the antigen preparations were used in the complement fixation test they were suitably diluted so that the first tube, in the test proper, contained 50 p.c. of the smallest dose that was anticomplementary. Further dilutions of antigen were made as indicated in Table 4. Fresh guinea-pig serum was used as the source of complement. Fixation of complement was allowed to take place for one hour at 37° C. then the haemolytic system (consisting of a 5 p.c. suspension of sheep's red blood cells sensitized with an equal volume of haemolysin diluted to contain 8 M.H.D.) was added, the test was incubated at 37° C. for one hour and was then allowed to stand in the refrigerator until the red cells had sedimented, after which the test was read. A four volume technique was used in the test and the total volume of fluid in each tube was 1 ml. consisting of 0·25 ml. of antigen, 0·25 ml. of complement, 0·25 ml. of serum and 0·25 ml. of sensitized red cells. Antigen, serum, and complement controls were included in the test. From the results of preliminary experiments carried out on the *C. equi* system a dilution of complement containing 2 M.H.D. was used in the complement fixation tests. A 1 in 5 dilution of serum was used throughout the test. Complement fixation was allowed to take place for one hour at 37° C.

RESULTS.

Cultural Examinations of the Submaxillary Lymph Nodes from Pigs.

The submaxillary glands of 41 pigs were examined culturally for the presence of *C. equi* and tubercle bacilli. Thirteen of these glands appeared normal, and macroscopically no lesions were detected. The remaining 28 glands appeared diseased and macroscopically showed definite, tuberculous-like lesions. The pigs from which these glands were taken, however, showed similar lesions in no other parts of the body. Sixteen strains of *C. equi* were isolated in all; the normal glands yielded one strain and 15 strains were obtained from the diseased glands. No tubercle bacilli were isolated from any of the 41 specimens studied.

Morphological and Cultural Characteristics of the Strains of C. equi.

The morphological, cultural and biochemical reactions of the 21 strains of *C. equi* included in this study were identical. All the organisms were markedly Gram-positive and coccobacillary in shape. In smears the organisms were arranged in the form of "Chinese letters" so characteristic of the *Corynebacteria*. They grew well on nutrient agar, producing large, circular, opaque, moist colonies with an entire edge. Often the colonies tended to coalesce because the growth was so moist. On standing at room temperature the organisms produced a characteristic bright, pinkish-tan pigment. In broth they grew well and produced an even turbidity. With long periods between subcultures several of the strains, including the type strain, *C. equi* Magnusson, showed what appeared to be a smooth to rough variation. The variant form on solid media produced small, discrete, granular colonies which were very difficult to emulsify. In broth, the variant form produced a granular deposit with no marked turbidity and often a granular, surface pellicle which readily broke up on shaking. However, with rapid subculture from solid to liquid media these "rough" organisms quickly reverted to their former "smooth" state.

All strains produced no fermentation of dextrose, maltose, sucrose, lactose, or mannite, after 7 days at 37° C., when grown in 1 p.c. peptone water containing 1 p.c. of the carbohydrate under test.

Serological Work.

The results of the precipitation tests are summarized in Table 1.

TABLE 1.

Precipitation tests.

Extracts of organisms	Undiluted antisera						
	C63	C65	Carne S.N.1321	Holth	Magnusson	Carne S.N.4563	C113
Strains of *C. equi* Group 1.							
C. equi Holth	—	+	—	+	—	—	—
C2	—	+	—	+	—	—	—
C65	—	+	—	+	—	—	—
C78	—	+	—	+	—	—	—
C111	—	+	—	+	—	—	—
C112	—	+	—	+	—	—	—
C115	—	+	—	+	—	—	—
C117	—	+	—	+	—	—	—
C139	—	+	—	+	—	—	—
C143	—	+	—	+	—	—	—
C214	—	+	—	+	—	—	—
C217	—	+	—	+	—	—	—
C218	—	+	—	+	—	—	—
Group 2.							
C. equi Magnusson	+	—	—	—	+	—	—
C. equi N.C.T.C. 1621	+	—	—	—	+	—	—
C63	+	—	—	—	+	—	—
C66	+	—	—	—	+	—	—
C216	+	—	—	—	+	—	—
Individual strains.							
C113	—	—	—	—	—	—	+
C. equi Carne S.N.4563	—	—	—	—	—	+	—
C. equi Carne S.N.1321	—	—	+	—	—	—	—
Diphtheroids used for comparison.							
D1, 2, 3, 4, 5, 6, 7	—	—	—	—	—	—	—

+ = ring present. — = no ring.

According to these tests the strains of *C. equi* did not form a homogeneous, serological group. However, the majority of the strains fell into one of two main groups, and there appeared to be no cross reaction between the organisms in the different groups. Extracts of 18 of the 21 strains of *C. equi* gave positive ring tests with either the C65 and Holth antisera or with the C63 and Magnusson antisera. The majority of these strains (13) proved to be Holth types, while only 5 of the strains were represented by the Magnusson type. The three remaining strains—*C. equi* Carne SN1321, *C. equi* Carne SN4563 and C113—appeared to be serologically distinct types reacting only with their homologous antisera, and by the precipitation technique, showed little antigenic relationship to the main types or to each other. The extracts of the diphtheroids used for comparison gave no reaction with any of the sera.

The results of the tube and slide agglutination tests are given in Tables 2 and 3 respectively.

TABLE 2.

Tube agglutination tests.

Suspensions of organisms	Antisera						
	C63	C65	Carne S.N.1321	Holth	Magnusson	Carne S.N.4563	C113
Strains of *C. equi* Group 1.							
C. equi Holth	—	160	—	40	—	—	—
C2	—	80	—	10	—	—	—
C65	—	160	—	20	—	—	—
C78	—	80	—	20	—	—	—
C111	—	40	—	10	—	—	—
C112	—	160	—	20	—	—	—
C115	—	80	—	20	—	—	—
C117	—	40	—	5	—	—	—
C139	—	80	—	10	—	—	—
C143	—	80	—	20	—	—	—
C214	—	160	—	10	—	—	—
C217	—	40	—	5	—	—	—
C218	—	40	—	5	—	—	—
Group 2.							
C. equi Magnusson	80	—	—	—	160	—	—
C. equi N.C.T.C. 1621	160	—	—	—	160	—	—
C63	160	—	—	—	160	—	—
C66	80	—	—	—	40	—	—
C216	40	—	—	—	40	—	—
Individual strains.							
C113	—	—	—	—	—	—	40
C. equi Carne S.N.4563	—	—	—	—	—	80	—
C. equi Carne S.N.1321	—	—	80	—	—	—	—
Diphtheroids used for comparison.							
D1, 2, 4, 5, 6	—	—	—	—	—	—	—
D3, 7	Suspension autoagglutinable						

Figures indicate the reciprocal end titre with different antigens.
— indicates no agglutination at 1 in 5.

The reactions observed were again highly type specific and agreed with the results of the precipitation tests. Here too there was very little, if any, cross reaction between the different types. By either slide or tube agglutination techniques, the strains were divisible into distinct groups identical with those demonstrated by the precipitin method. It was impossible however, to determine whether the strains that fell into each of these different groups were antigenically identical without resorting to reciprocal agglutinin absorption tests. None of the diphtheroids used for comparison gave any reaction with the sera, but unfortunately, the behaviour of two of the strains, D3 and D7, was not determined because the organisms were auto-agglutinable when suspended in normal saline. However, these two strains gave no reaction by the precipitin method, so it was unlikely that they would have been agglutinated by the sera had the suspensions been stable.

The results of the complement fixation tests are given in Table 4.

TABLE 3.

Slide agglutination tests.

Suspensions of organisms	Undiluted antisera						
	C63	C65	Carne S.N.1321	Holth	Magnusson	Carne S.N.4563	C113
Strains of *C. equi* Group 1.							
C. equi Holth	—	++++	—	++++	—	—	—
C2	—	++++	—	++++	—	—	—
C65	—	++++	—	++++	—	—	—
C78	—	++++	—	++++	—	—	—
C111	—	++++	—	++++	—	—	—
C112	—	++++	—	++++	—	—	—
C115	—	++++	—	++	—	—	—
C117	—	++++	—	++++	—	—	—
C139	—	++++	—	++++	—	—	—
C143	—	++++	—	++++	—	—	—
C214	—	++++	—	++	—	—	—
C217	—	++++	—	++	—	—	—
C218							
Group 2.	++++	—	—	—	++++	—	—
C. equi Magnusson	++++	—	—	—	++++	—	—
C. equi N.C.T.C. 1621	++++	—	—	—	++++	—	—
C63	++++	—	—	—	++++	—	—
C66	++++	—	—	—	++++	—	—
C216							
Individual strains.	—	—	—	—	—	—	++++
C113	—	—	—	—	—	++++	—
C. equi Carne S.N.4563	—	—	++++	—	—	—	—
C. equi Carne S.N.1321							
Diphtheroids used for comparison.	—	—	—	—	—	—	—
D1, 2, 4, 5, 6	—	—	—	—	—	—	—
D3, 7			Suspension autoagglutinable				

++++ = Complete agglutination.
+, ++, +++ = Intermediate degrees of agglutination.
— = No agglutination.

All the strains of *C. equi* fixed complement in the presence of the antiserum prepared against *C. equi* Magnusson while the other diphtheroids gave no fixation of complement, under the conditions of the test. The strains of *C. equi* must therefore possess a common antigenic component which apparently is not present in the diphtheroids used for comparison. Bruner and Edwards (1941), who have studied the serology of *C. equi* very extensively, believe that a species-specific antigen is responsible for this complement fixation, and that this antigen is only laid bare after the organisms have been treated with acid and thus freed of the soluble, type-specific substance which normally masks this antigen. The organisms then no longer react as individual types (as they do in the precipitation and agglutination tests), but as a group. This result indicates the presence of a species-specific antigen common to all the strains.

TABLE 4.

Complement fixation tests.

Residue of organisms after acid extraction	Magnusson antiserum				
	Antigen dilutions				
	1	1/2	1/4	1/8	1/16
C. equi strains					
C. equi **Magnusson**	++++	++++	++++	++++	+++
C. equi **N.C.T.C. 1621**	++++	++++	++++	++++	++
C. equi **Holth**	++++	++++	++++	++++	+
C. equi **Carne S.N.1321**	++++	+++	±	0	0
C. equi **Carne S.N.4563**	++++	++++	+	0	0
C2	++++	++++	++	++	0
C63	++++	++++	++++	+++	+++
C65	++++	+++	±	0	0
C66	++++	++++	++++	++	0
C78	++++	++++	+++	0	0
C111	++++	++++	+++	0	0
C112	++++	++++	+	0	0
C113	++++	++++	+	0	0
C115	++++	++++	+	0	0
C117	++++	+++	+	0	0
C139	++++	++++	++++	++++	+++
C143	++++	++++	++++	++++	+
C214	++++	+++	±	0	0
C216	++++	++++	++++	++++	++
C217	++++	++++	+	0	0
C218	++++	++++	++++	++	0
Diphtheroids used for Comparison					
D1, 2, 3, 4, 5, 6, 7	0	0	0	0	0

++++ = Complete fixation of complement (i.e. no haemolysis).
+++, ++, +, ± = Intermediate degrees of fixation (i.e. partial haemolysis).
0 = No fixation of complement (i.e. complete haemolysis).

While by the results of the precipitation and agglutination tests the 20 strains of diphtheroids studied here did not appear to form a homogeneous serological group, the results of the complement fixation tests demonstrated their relationship to each other and to the type strain, *C. equi* Magnusson, and identified them as strains of *C. equi*.

DISCUSSION.

The present study has confirmed the observations of Holth and Amundsen (1936), Bendixon and Jepsen (1938), Jespersen (1938a), Plum (1938, 1939 and 1940 a and b), Magnusson (1940), Feldman, Moses and Karlson (1940), McDonald (1942) and Cotchin (1943) on the occurrence of *C. equi* in the lymph glands of the head and neck in pigs. However, the majority of these workers identified their cultures as strains of *C. equi* on morphological, cultural and biochemical characters, and identification on these grounds alone

often proves very difficult and may be unreliable. Karlson, Moses and Feldman (1940) at first identified 29 chromogenic diphtheroids, on their cultural characteristics as strains of *C. equi*, but 3 of them on antigenic analysis proved not to be *C. equi* types. Serological procedures to substantiate the cultural findings were employed only in the present study and by Bruner, Dimock and Edwards (1939), Magnusson (1940), Feldman, Moses and Karlson (1940). Unfortunately, Cotchin (1943) was unable to investigate his strains of *C. equi*, isolated from pigs, by serological techniques because he could not produce satisfactory antisera.

In the present study, sixteen strains of *C. equi* were obtained from the 41 specimens examined. Thirteen of these glands, on gross examination, appeared normal, and from them only 1 strain of *C. equi* was isolated. The remaining 28 glands showed typical tuberculous-like lesions, and from them the other 15 strains were recovered. In none of the specimens, as far as the present tests went, were the strains of *C. equi* associated with viable tubercle bacilli. These results agree with those of Jepsen (1940). In his study of material from 400 diseased pigs, 177 strains of *C. equi* and 180 strains of tubercle bacilli were isolated and only on five occasions were the two organisms isolated together. However, Feldman, Moses and Karlson (1940) found that the majority of their strains of *C. equi* were associated with tubercle bacilli. From 89 diseased glands, 20 strains of *C. equi* were obtained. In 13 glands *C. equi* was associated with viable tubercle bacilli, and in only 7 glands did *C. equi* occur alone.

The present study in its limited extent, has also shown that the incidence of *C. equi* in apparently normal gland tissue is low. Only one strain was isolated from 13 normal glands examined, that is, the organism was isolated from only 7·7 p.c. of the specimens. Feldman, Moses and Karlson (1940), on the other hand, recovered 6 strains of *C. equi* from 25, apparently normal submaxillary lymph nodes, so the organism was isolated from 24 p.c. of their specimens. They also found that the organisms occurred just as frequently in normal glands as they did in diseased glands, in fact they recovered 20 strains from 89 diseased glands, which means that the organisms occurred in 22·4 p.c. of the specimens examined. The results of the present study do not agree with the above findings—while *C. equi* only occurred in 7·7 p.c. of the normal glands examined, 15 strains were recovered from 28 diseased glands —an incidence of 53·6 p.c.

When the agglutination and precipitation reactions of the strains of *C. equi* included in the present study were examined, it was evident that within the species there were several distinct antigenic types. In this respect the work confirmed the observatons of Rajagopalan and Gopalakrishnan (1939) who found that cross agglutination tests between a strain of *C. equi* isolated from a foal, and an unidentified strain isolated from a buffalo-cow, and their homologous antisera were negative, and the relationship of the

strains was only demonstrated later by means of a complement fixation test, using acid-treated organisms. Bruner and Edwards (1941) likewise showed that the 34 cultures which they examined were not antigenically homogeneous, but included a number of distinct types. Karlson, Moses, and Feldman (1940) also proved the same point. Only 4 of their 26 strains isolated from pigs were agglutinated by a Magnusson antiserum. In the present study, too, only 3 out of 16 strains locally isolated from pigs showed agglutination or precipitation with a Magnusson antiserum: the number of strains represented by this type was in a minority. Magnusson (1938) on the contrary, found that his strains constituted a homogeneous, serological group, and all were agglutinated by the same antiserum. However, all but 2 of his strains were isolated during a single outbreak of disease, probably all originated from the same source, and were therefore likely to provide only one serological type. The strains studied here and by other workers, were "single isolations" and the chances were that, in their origin, they were completely unrelated to each other.

In the present study the majority of the 21 strains were represented by two distinct serological types—*C. equi* Holth and *C. equi* Magnusson. Also cultures isolated in America and India proved to be the Magnusson type, so the distribution of this type appears to be world wide. Three of the Australian strains of *C. equi* appeared to be distinct serological types not previously recorded.

No matter what the serological type or the source of the strains, a complement fixation test using acid-treated organisms established their relationship with each other. In this respect the results confirmed the observations of Rajagopalan and Gopalakrishnan (1938), Bruner, Dimock and Edwards (1939) and Karlson, Moses and Feldman (1940). Thus 16 cultures isolated locally from the submaxillary lymph nodes of pigs, and cultures from the pneumonic lungs of foals and from the nephritic kidney of an 8-year-old mare were all identified as strains of *C. equi*.

SUMMARY.

The submaxillary lymph glands from 41 pigs were examined culturally for the presence of *C. equi* and tubercle bacilli. Thirteen of the glands, on macroscopic examination, appeared normal. The remaining 28 glands showed typical, tuberculous-like lesions. Sixteen strains of *C. equi* were obtained. No tubercle bacilli were isolated in culture from any of the lymph glands examined.

The morphological, cultural, biochemical and serological characteristics of these 16 strains, together with 5 other strains of *C. equi* not locally isolated, were studied.

The pinkish-tan, confluent growth of the organisms on solid media was characteristic.

Complement fixation tests, using acid-extracted organisms, indicated the presence of a common species-specific antigen and identified the 21 cultures as strains of *C. equi.*

Agglutination and precipitation tests indicated the presence of type-specific antigens. Thirteen of the strains were agglutinated or precipitated by the antiserum prepared against *C. equi* Holth. Five of the strains reacted similarly with the antiserum prepared against *C. equi* Magnusson. The 3 remaining strains were not agglutinated or precipitated by either of these antisera and were classed as distinct antigenic types.

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SMALL COLONY VARIANTS ("G" FORMS) PRODUCED BY *STAPH. PYOGENES* DURING THE DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN

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Hoffstadt and Youmans (1932) isolated small colony variants, "G" forms, from *Staphylococcus aureus* by growing the organisms in 0·5 p.c. lithium chloride in beef infusion broth and by animal inoculation. Youmans and Delves (1942) used barium chloride or barium nitrite in peptone water to select "G" types, while Hale (1947) employed M/15 barium chloride in 1 p.c. peptone water and nutrient agar plates containing 1 part per million of gentian violet.

Hoffstadt and Youmans (1932) reported that the "G" colonies were small, translucent and bluish in colour. The "G" form fermented readily all sugars fermented by other types. Hale (1947) stated that the "G" variants had lower metabolic activities than the parent strains. He found that whereas the parent strain produced acid from glucose, lactose and sucrose after 18 hours' incubation at 37° C., the "G" form produced acid only from glucose after 48 hours' incubation. Similar observations were recorded by Swingle (1935).

EXPERIMENTAL.

During the *in vitro* development of resistance of streptomycin by five strains of *Staphylococcus pyogenes* (Barbour, 1950) small colony variants, similar to "G" types were observed. All five strains produced these variants. Platings on blood agar from strain R15 growing for the first time in 0·005, 0·01, 0·015 and 0·1 mg. of streptomycin per ml. of broth and incubated for 24 hours revealed a higher proportion of the variant form than the normal colonial type. Platings from all five strains growing for the first time in 100 mg. of streptomycin per ml. of broth showed a proportion of the "G" form but after a further one or two passages through this concentration of the antibiotic platings revealed only the normal colony type.

The variant colonies were of pin-point size, translucent, unpigmented and non-haemolytic. In contrast to the observations of Hoffstadt and Youmans (1932) the small colony variants were of uniform size. Morphologically and in staining reactions the cells from the "G" colonies resembled those from the parent strains.

A more detailed study was made of the variant obtained from a plating of strain R185 growing in 50 mg. of streptomycin per ml. of broth. Growth in glucose-veal broth, although

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much slower than that of the parent form, reached approximately the same final turbidity. Similarly, the growth rate on blood agar plates and on nutrient agar slopes was much less than that of the parent. On nutrient agar slopes the variant produced a scarcely visible film of growth.

The "G" form retained its cultural characteristics and resistance to 50 mg. of streptomycin per ml. of broth after 6 serial sub-cultures in the absence of the antibiotic.

Although the "G" form resembled its parent strain in fermenting glucose, lactose and maltose, it did so much more slowly, requiring 48 hours at 37° C. to produce an acid reaction compared with 18 to 24 hours for the parent. Although the parent strain produced a slightly acid reaction in mannitol after 24 hours' incubation, the "G" form would not grow in the presence of this sugar, despite repeated attempts using inocula of varying sizes.

Both parent and variant were coagulase-positive. They were both lysed by phage 31B (Rountree and Thomson, 1949), but, in order to obtain confluent lysis of the "G" form by the normal phage plating technique, it was necessary to use a very heavy inoculum of bacteria to obtain a continuous film of growth on the agar plate. It was therefore of interest to determine if there were any differences between the burst sizes and growth rates of phage growing in the "G" variant and in the parent strain.

A one-step growth curve of phage 31B in the "G" variant was established by the following method:

> Equal volumes of phage of known concentration and of a 4-hour culture of the "G" form in glucose-veal broth to which had been added 20 μg. of tryptophan per ml. and 0·02 p.c. calcium chloride were mixed and incubated for 20 minutes at 37° C. to allow adsorption of the phage onto the bacterial cells. At the end of this time the mixture was diluted 1/10,000 in broth to which had been added 20 μg. tryptophan per ml. and 0·02 p.c. calcium chloride. The diluted mixture, which was estimated to contain approx. 1,000 phage particles/ml., was incubated at 37° C. and from it duplicate platings of 0·01 ml. volumes were made on quadrants of nutrient agar plates on which had been spread the propagating strain of phage 31B. The platings were made at the intervals indicated in Fig. 1. The plates were incubated at 30° C. overnight and plaque counts made the following morning.

A similar experiment was carried out to establish the growth curve of phage 31B in the parent strain, R185.

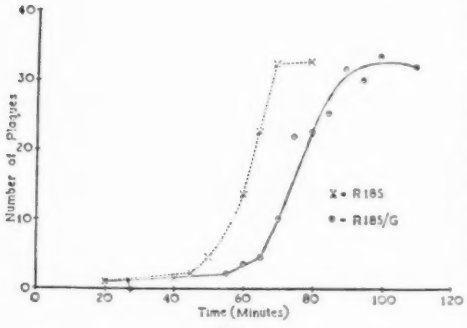

Fig. 1.

In plotting the curves shown in Fig. 1, the actual counts obtained have been converted to a basis of the yield from an average of one infected bacterium. The results show that there has been no reduction in the average yield of phage particles from infected cells of the variant, both strains giving an average yield of 32 per cell. The release of phage from the infected cells of the parent strain commenced at 45 minutes and was complete in 70 minutes, whereas with the "G" form the first release of phage was detected at 60 minutes and complete release was not accomplished until 90 minutes had elapsed. There was, therefore, a considerable slowing-down of the rate of phage production in the "G" form.

DISCUSSION

Hale (1947) found that it was necessary to incubate staphylococci for from 5 to 7 days in barium chloride-peptone broth before obtaining "G" variants. Using the method of plating on nutrient agar containing 1 p.p.m. of gentian violet "G" colonies were observed within 42 to 72 hours.

Hoffstadt and Youmans (1932) reported that 38 serial sub-cultures in lithium chloride-beef infusion broth were necessary before it was possible to isolate a stable "G" variant. In contrast to these observations, platings from 24-hour broth cultures containing streptomycin revealed a high proportion of "G" colonies. It is apparent that streptomycin has a stronger selective action for the "G" variants than have lithium chloride, barium chloride or gentian violet. The nature of this selective action is obscure, although it is possible that the "G" forms are naturally more resistant to the antibiotic than are the parent types. This would be extremely difficult to prove as the parent strain also produces normal colony types which are resistant to streptomycin.

The observations on the reduced metabolic rate of the "G" form compared with that of the parent strain are in agreement with those of Swingle (1935) and Hale (1947). However, whereas both Hale and Swingle stated that the "G" forms studied by them fermented only glucose, the variant reported in this paper fermented glucose, lactose and maltose, although much more slowly than the parent strain. Swingle found that lactose and sucrose had an inhibitory action on the growth of the "G" form, whereas mannitol appeared to have a similar inhibitory action on the variant described in this paper.

Hale reported that his "G" variants and their parent strains were essentially of the same serological and phage types. The "G" variants examined here were also of the same phage type as the parent strains. The lowered metabolic rate of the "G" variant was, however, reflected in the reduced rate of phage production in infected cells, although the average yield of phage particles per cell was not affected. It has not been possible to examine these strains serologically but since it has been shown elsewhere that lysis of rough variants by similar type specific bacteriophages does not occur (Rountree, 1947) it is legitimate to assume that the "G" mutation studied here is one which does not involve loss of the smooth antigens of the staphylococcus. Similarly, the ability to coagulate plasma has been retained.

SUMMARY.

Small colony variants ("G" forms) were observed during the *in vitro* development of streptomycin resistant strains of *Staphylococcus pyogenes*. The colonies were of pin-point size, unpigmented and non-haemolytic.

Isolation and further study of a "G" form indicated that its metabolic rate was much lower than that of its parent strain. Glucose, lactose and maltose were fermented by both the parent and variant strains but the latter required 48 hours to produce an acid reaction compared with 18–24 hours required by the former. Mannitol appeared to have an inhibitory action on the growth of the "G" form.

Both the parent strain and the "G" variant were lysed by phage 31B. One step growth curves of this phage in the parent and the "G" form indicated that phage was produced more slowly by the variant than by the parent strain but that the average number of phage particles produced per cell was of the same order in both cases.

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THE DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN BY *STAPHYLOCOCCUS PYOGENES*

by R. G. H. BARBOUR[1]

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Various workers have studied the development of resistance to streptomycin *in vitro* by *Staph. pyogenes*. Demerec (1948) used one strain of *Staph. pyogenes* to examine this problem. He drew attention to the stepwise increase in degree of resistance that can be brought about by selection and noted that the building up of resistance is more rapid with each selection step. He observed that this building up of resistance showed a considerable degree of variability. For example, from first step resistant cultures there could be isolated colonies that were only slightly more resistant than the original strain, some that were almost completely resistant and some that fell between the two extremes. This variability was exhibited in all steps.

Suter and Vischer (1948) increased the resistance *in vitro* of 31 freshly isolated strains of staphylococci and found the resistant variants to be just as virulent as their parent strains when injected intraperitoneally into mice.

The work reported in this paper on the development of resistance to streptomycin by *Staph. pyogenes* attempts to correlate the results of experiments carried out *in vitro* with observations on the development of resistance *in vivo* and to compare the characteristics of the *in vivo* and *in vitro* resistant strains.

EXPERIMENTAL.

The Development of Streptomycin Resistance in Vitro.

Materials and Methods.

The *in vitro* development of resistance to streptomycin by three partly resistant and two sensitive strains of *Staph. pyogenes* has been studied. The characteristics of the five strains, all of which were isolated from patients in the Royal Prince Alfred Hospital, are given in Table 1.

The degree of resistance of a strain was determined by inoculating a series of concentrations of streptomycin in 1 ml. of glucose-veal broth with 0·01 ml. of an 18-hour broth culture

[1] This work was carried out under a grant from the Australian National Health and Medical Research Council.

of the strain diluted 1/100 (approximately 1 × 10^5 organisms). After 24 hours' incubation at 37° C. the highest concentration of the antibiotic in which visible turbidity was observed was recorded as the resistance of that strain.

TABLE 1.

Origin and characteristics of five strains of Staphylococcus pyogenes *used in the examination of the* in vitro *development of resistance to streptomycin.*

Strain number	Source of strain	Streptomycin resistance (mg./ml.)	α haemolysin production	Coagulase reaction
R15	P.M. intestine	0·001	+	+
R194	Osteomyelitis of skull	0·001	+	+
R40	Bedsore	0·20	+	+
R165	Osteomyelitis of skull	1·00	+	+
R185	Abscess	0·50	+	+

Increased resistance was developed by inoculating a series of streptomycin concentrations with 0·01 ml. of undiluted cell suspension (approximately 1 × 10^7 organisms) from that tube containing the highest concentration of the antibiotic to which the organism was previously resistant. After 24 hours' incubation at 37° C. the new resistance range was recorded and the process repeated.

Results.

It can be seen from Fig. 1 that conversion of a partly resistant strain to one which is completely resistant is accomplished extremely rapidly. With sensitive strains the resistance increases by relatively small steps until a threshold resistance of approximately 0·1 mg./ml. of broth is reached, after which completely resistant variants quickly appear.

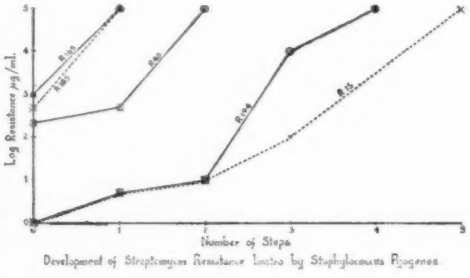

Fig. 1.

Five strains of which two had been made resistant *in vitro* and three which had become resistant *in vivo* and which grew in 100 mg. of streptomycin per ml. were tested to determine if they inactivated streptomycin in a manner analagous to the inactivation of penicillin by penicillin-resistant staphylococci. After incubating the strains for 24 hours at 37° C. in the presence of 1 mg. of the antibiotic per ml. of broth the cultures were centrifuged at 4,000 r.p.m. for 15 minutes. The supernatants were tested for loss of antibiotic activity. A control of streptomycin in uninoculated broth, incubated at 37° C. for 24 hours, was included in each test. 0·02 ml. quantities of the supernatants were placed on filter paper discs in the centre of blood agar plates which were then inoculated by streaking with the Oxford H-strain of *Staph. pyogenes*. Comparing the areas of inhibition obtained with the control and with the supernatants from

the streptomycin resistant cultures, no evidence could be seen of any inactivation of the streptomycin in these cultures.

Staphylococci made resistant *in vitro* have remained resistant over a period of 6 months involving approximately 15 subcultures in the absence of the antibiotic.

Sugar fermentation reactions, liquefaction of gelatin, coagulase reaction, haemolysin production and bacteriophage type were determined and, with one exception, no differences between resistant variants and their parent strains were found. The one exception was the resistant strain of R165 which liquefied gelatin while the parent strain did not. These results are recorded in Table 2. In all cases both parent and resistant variant were coagulase-positive and alpha-haemolytic.

In morphology and staining reactions the resistant variants were identical with the strains from which they were derived.

TABLE 2.

Metabolic characters of staphylococci made resistant to streptomycin in vitro *and of their parent strains.*

Strain number	Sugar fermentation				Gelatin Liquefaction	Coagulase	Haemolysin	Phage type
	Lactose	Glucose	Mannitol	Maltose				
R15 (Parent)	A	A	A	A	+	+	+	31B
R15 (Resistant)	A	A	A	A	+	+	+	31B
R194 (Parent)	A	A	A	A	+	+	+	7/42B/47C
R194 (Resistant)	A	A	A	A	+	+	+	7/42B/47C
R40 (Parent)	A	A	±A	A	+	+	+	31A/31B
R40 (Resistant)	A	A	±A	A	+	+	+	31A/31B
R165 (Parent)	A	A	—	A	—	+	+	31B
R165 (Resistant)	A	A	—	A	+	+	+	31B
R185 (Parent)	A	A	±A	A	—	+	+	31B
R185 (Resistant)	A	A	±A	A	—	+	+	31B

A = production of acid after 24 hours at 37° C. ±A = slight acid production after 24 hours at 37° C. — = no change. Gelatin liquefaction—4 days at 37° C.

The Development of Streptomycin Resistance in Vivo.

During the period 1st June to 15th September, 1949, streptomycin resistant staphylococci were isolated from infected burns or wounds in 17 patients. Of these patients, 8 had received no streptomycin therapy and their organisms had been acquired as the result of hospital cross infection, while of the remaining 9, three had received streptomycin by intramuscular injection and 6 by local application. These observations are summarized in Table 3.

TABLE 3.

Analysis of 17 cases yielding streptomycin resistant Staph. pyogenes.

Origin of resistant strain	Streptomycin therapy		No streptomycin
	Intramuscular injection	Local application	
Conversion to resistance *in vivo* Hospital cross infection	3	6	8

The degree of streptomycin resistance of the organisms isolated is recorded in Table 4.

TABLE 4.

Range of streptomycin resistance of 17 strains of staphylococci isolated from patients.

Streptomycin resistance in mg./ml.	Source of resistant strains		
		Streptomycin therapy	
	Cross infection	Local application	Intramuscular injection
0·1	2	—	1
0·5	—	1	—
1·0	3	1	1
10·0	2	—	1
50·0	—	1	—
100·0	1	3	—
Total	8	6	3

The resistant strains were of 3 phage types: 31B, 47/31B and 47. All were also penicillin resistant.

It may be of interest to cite an example of the development of resistance *in vivo*. A patient was admitted to the hospital on 16th July suffering from burns. On the 1st August a swab showed *Staph. pyogenes*, phage type 47/31B, which was penicillin resistant and streptomycin sensitive. Local streptomycin was given and on the 16th August a swab showed *Staph. pyogenes*, phage type 47/31B, which was penicillin resistant but now resistant to 0·5 mg. of streptomycin per ml. A swab taken a week later indicated that the organism had become resistant to 1·0 mg. of streptomycin per ml.

No cultural or morphological differences could be detected between the strains made resistant *in vivo*, those made resistant *in vitro* and streptomycin sensitive strains of the same phage types isolated from other patients during the investigation.

DISCUSSION

It appears that streptomycin resistant strains can be built up *in vivo* as rapidly as *in vitro*. The experiments carried out *in vitro* suggest that once a strain has attained a certain threshold of resistance further application of streptomycin will result in the rapid building up of a highly resistant variant.

It may well be that the degree of resistance reached is related to the concentration of streptomycin to which the organism has been exposed. The higher resistance of staphylococci isolated from cases treated locally with the antibiotic may be explicable by the fact that these organisms have been exposed to higher concentrations of streptomycin than are attained by intramuscular injection. (Table 4).

Marked changes in morphology and staining reactions have been reported for staphylococci made resistant to penicillin *in vitro* (Graessle and Frost, 1946; Bellamy and Klimek, 1948; Gale and Rodwell, 1949). Gale and Rodwell (1949) state that the degree of penicillin resistance of *Staphylococcus aureus* is determined by the extent to which its growth processes are independent of the assimilation of preformed amino-acids. However, Barber (1949), reviewing work on penicillin resistance of staphylococci, points out that organisms which have become resistant *in vivo* produce a powerful penicillin inactivator and that such staphylococci do not otherwise differ from typical penicillin sensitive strains in morphology, cultural characteristics or virulence.

In contrast to the observations on staphylococci made resistant to penicillin *in vitro* the work reported in this paper shows that the development of streptomycin resistance *in vitro* was not accompanied by changes in morphology or staining reactions. Furthermore, with one exception, no change could be detected in the range of enzyme reactions examined. Strains made resistant *in vitro* did not appear to differ from those which had developed resistance *in vivo*. Both types of resistant variant retained the ability to produce haemolysin and coagulase, which indicates their pathogenicity. In contrast to the penicillinase-producing staphylococci no inactivation of streptomycin by either type of resistant variant could be demonstrated.

Streptomycin resistant staphylococci obviously, by definition, differ from streptomycin sensitive strains in at least one characteristic but this study has not revealed the basis of this difference.

SUMMARY.

Five strains of *Staphylococcus pyogenes*, three partly resistant and two sensitive to streptomycin, were made resistant *in vitro* to 100 mg. of streptomycin per ml. Conversion of the partly resistant strains to complete resistance was accomplished extremely rapidly. The resistance of the sensitive strains increased by relatively small steps until a threshold of resistance of approximately 0·1 mg/ml. was reached, after which completely resistant variants quickly appeared.

No inactivation of streptomycin could be detected either by strains made resistant *in vitro* or by those which had become resistant *in vivo*.

Comparison of parent strains and their resistant variants did not reveal any consistent differences in morphology, staining reactions, enzyme reactions, haemolysin and coagulase production or bacteriophage type.

Strains made resistant *in vitro* did not appear to differ from those which had become resistant *in vivo*. Both types of resistant variant retained the ability to produce haemolysin and coagulase, which indicates their pathogenicity.

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VAGAL REFLEX INFLUENCES ON CENTRAL RESPIRATORY CO-ORDINATION

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A variety of vagal afferent endings presumably possessing the common property of being activated by changes in lung volume, are found in the epithelia of the lung structures. The relationship of the afferent discharge frequencies of these endings to the phases of respiration suggests that during the inspiratory period the vagi conduct pulmonary afferent impulses of increasing frequency which summate centrally and contribute in part to the initiation of expiration; the overall effect being one of shortening both the inspiratory and expiratory phases of respiration (Adrian, 1933; Partridge, 1933, 1935; Rijlant, 1933).

The Hering-Breuer concept of respiratory control as elaborated by Head (1889) requires two vagal reflexes. One is inspiratory inhibitory on lung inflation, the other expiratory inhibitory on lung deflation, and although Knowlton and Larrabee (1946) have confirmed the earlier observations on two vagal fibre types concerned in respiration, the fibre groups having different thresholds and adaptations, there does not seem to be any agreement as to the origin or mechanism of the accelerating effect of lung deflation (Partridge, 1939; Hammouda and Wilson, 1932 to 1939; Steffensen *et al.*, 1937). Suction deflation, at best an unphysiological procedure, may stimulate endings not normally associated with the Hering-Breuer reflex. Histologically, the lungs contain endings of varied appearance possibly capable of activating several different reflexes, unless they all respond to the common stimulus of lung deformation (Elftman, 1943). The fact that low frequency stimulation of the central vagal stump will produce acceleration of respiration while high frequencies are inhibitor (Rice, 1938), has been taken up by Wyss (1946a). He has shown that the frequency conditioned reflex reversal is quite similar to, and even simpler than, that described for spinal reflexes, in that internuncial neurones mediate their excitor or inhibitor effects upon the respiratory motor neurones by virtue of differences in summative capacity. These effects are relayed by discrete regions within the medulla in the tractus solitarius and adjacent parts of the lateral reticular formation (Wyss, 1946b), while the extent of their actions on the respiratory rhythm is moderated by a system which extends into the upper pons (Meier and Bucher, 1941; Bucher, 1942), since serial sectioning of the brain stem caudad from the lower portion of the inferior colliculi releases first the excitor then, at a lower level, the inhibitor actions of the vagal reflexes so that they become more exaggerated.

[1] Supported by a Commonwealth Research Grant and the Sheridan Research Fund.

It is possible to obtain some functional differentiation of these reflexes in the intact brain stem. Deep ether anaesthesia produces a depression of both vagal reflexes. Barbiturates cause an exaggeration of both reflexes until the plane of anaesthesia is deep, when a heightened sensitivity of the inspiratory inhibitory reflex alone remains, the reflex behaviour resembling that which in Bucher's experiments resulted from the lower pontine sections (Gordh, 1945). During experiments on the respiratory responses to the nicotine group of drugs, variations in the respiratory pattern were frequently encountered, and it was further noticed that removal of the vagal influences generally complicated the respiratory picture, rather than making it more simple as might have been expected. The results suggest that the vagal influence on respiration is associated in part with the modification of the rhythm by altering the duration of both the inspiratory and expiratory phases, and in part with the tonic activity of the muscle groups participating in the respiratory act. This latter feature has not received so much attention as the rhythmic aspect, so that it seems desirable to detail some of the changes in the pattern of response of the respiratory muscles which occur on sectioning the vagi, and to comment on the relation of these results to the accepted views of activity within the respiratory centre.

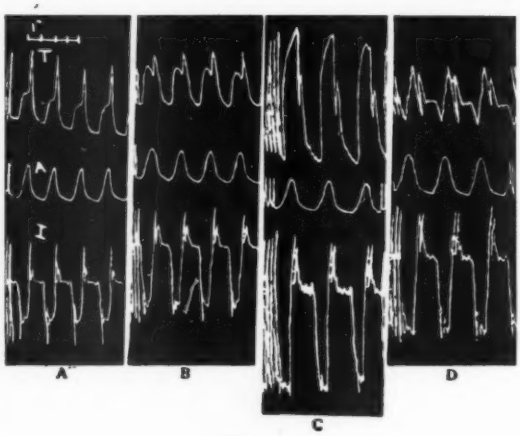


Fig. 1.

Fig. 1. Rabbit, paraldehyde anaesthesia. In this and subsequent records, except Figs. 4 and 5, T = Thoracic record, A = Abdominal record (both inspiration upwards), I = Intratracheal pressures (inspiratory negative pressure down, expiratory positive pressure upwards).

A. Normal respiration, during recovery from 10 p.c. CO_2. V.R. (Ventilation Rate) = 0·9 litres/min. The reflection of diaphragmatic retraction appears at end-inspiration on sustained thoracic activity.

B. Quiet respiration; V.R. = 1·1 litres/min. Between A and B both vagi were sectioned. Premature thoracic relaxation appears in late inspiration.

C. During subsequent administration of 10 p.c. CO_2; V.R. = 1·3 litres/min. The premature thoracic relaxation is more pronounced with the reflection of diaphragmatic retraction appearing on the "expiratory" limb.

D. "Double Thoracic Rhythm" during recovery from 10 p.c. CO_2; V.R. = 1·2 litres/min.

EXPERIMENTAL.

Methods.

Rabbits, anaesthetized with paraldehyde (1·7–1·8 ml./kg. p.o.) were used. The animals were prepared by insertion of a tracheal cannula. Subsequent procedures have been indicated in the text.

Respiration was recorded, in the supine position, by heavy condom tambours held in brass formers supported off the animal by carriers on which the animal lay. The recorders came in contact with the ventral abdominal and thoracic surfaces only by virtue of light inflation, thus offering minimal resistance to the respiratory movements.

The phases of respiration were followed by the pressure changes occurring between the valves of a Gaddum (1941) respiratory minute volume recorder attached to the tracheal cannula. The valves were modified from the original design. The valve leaf was a ¾-inch microscope cover slip resting on a glass optical flat through which the air flowed by an excentric 2 mm. hole drilled through the flat. The whole was mounted in a glass carrier set at an angle, the flap hinging on the lower carrier surface with the entrance hole at the top. The exit valve was heated by a 25 watt mains lamp to prevent the flap sticking to the seating through moisture condensation. The valves required about 1 mm. water pressure to be opened and showed no back leakage. Alternative air and CO_2 intakes were provided.

The pressure changes in the recording systems were registered either on a long-paper kymograph or optically on 12 cm. paper.

Results.

Interactions of the Respiratory Muscle Groups.

The respiratory patterns, as recorded by the present method, closely resemble those of Gordh (1945, Figs. 2, 3, 6 and 7). The thoracic diameter changes during each respiratory phase often record as a more complex pattern than do those from the abdomen. This complexity depends for its appearance upon the depth of anaesthesia and the functional state of the central and reflex respiratory mechanisms.

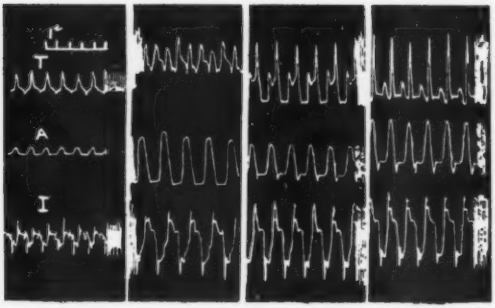
In quiet respiration with light anaesthesia the thoracic record characteristically shows a sharp deflection, in the inspiratory sense, superimposed upon the general diameter trend at the initiation of expiration as judged from the intra-tracheal pressures (Fig. 1 a). This deflection is coincident with the moment of diaphragmatic relaxation at expiration and is referred to by Gordh as a "paradoxical short, snappy elevation of the upper thorax at the moment of diaphragm retraction during expiration." Its appearance can be used as an indication of the cessation of diaphragmatic activity.

The thorax and abdomen patterns with the aid of this indicator of the diaphragmatic actions, can be taken to show that the respiratory centre controls the muscle groups in each respiratory act with some degree of independence at the thoracic and diaphragmatic levels.

The thorax may show:

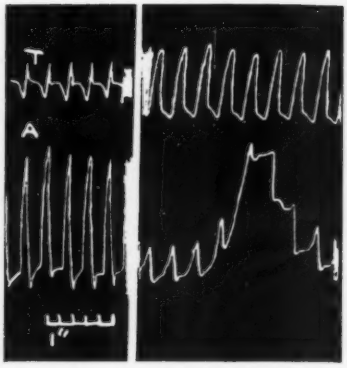
(a) Little inspiratory expansion but adequate fixation (Fig. 4a).
(b) Definite inspiratory expansion throughout the inspiratory phase (Fig. 1a).
(c) Poor fixation, resulting in a lessening of thoracic diameter which becomes more apparent as inspiration proceeds. This occurs usually after ether or on vagotomy (Fig. 3a).
(d) Intergrades of the preceding (Figs. 5a and b).

Some evidence of the diaphragmatic expiratory retraction is reflected to a greater or less extent onto these thoracic patterns. Increased respiratory activity (as with 10 p.c. CO_2 administration or chemireflex drive) tends to make it more apparent (Figs. 4b and 5a and b).



A B C D

Fig. 2.



A B

Fig. 3.

Fig. 2. Thoracic complexes during nicotine hyperpnoea. Rabbit, paraldehyde anaesthesia.

A. Normal respiration, V.R. = 0·5 litres/min.

B. During recovery from 1 mg. l-nicotine *i.v.*; V.R. = 0·75 litres/min. Between A and B both vagi were sectioned.

C. During a further hyperpnoea (1 mg. l-nicotine, 30 mins. after B), V.R. = 1 litre/min.

D. Later in the same response, V.R. = 0·9 litres/min.

The thoracic activity altered during the second nicotine response, true thoracic inspiratory activity became less and the reflection of diaphragmatic retraction greater from C to D. The nicotine dose approaches the lethal one and thoracic respiration failed, followed by complete respiratory arrest after a further 1 mg. of the drug.

Fig. 3. Rabbit, paraldehyde anaesthesia. Ether was given during operative preparation.

A. "See-saw" respiration in the normal animal, V.R. = 0·8 litres/min. This is similar to Gordh's thoracic pattern in ether anaethesia.

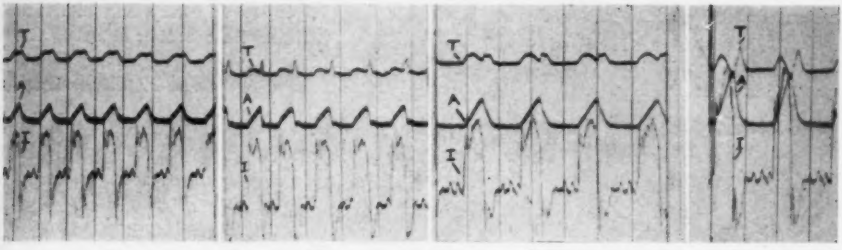
B. Quiet respiration, V.R. = 0·7 litres/min. Between A and B both vagi were sectioned, and the phrenics crushed near their origin. The thoracic record is uncomplicated even during increased respiratory activity. Peristaltic movements cause the shifting base line of the abdominal record.

Vagotomy and Consequent Changes in Respiratory Pattern.

Bilateral vagotomy at the cervical level consistently causes the inspiratory and expiratory periods to be lengthened. The intratracheal pressures show (Figs. 4 and 5) that after vagotomy the inspiratory negative pressure declines toward end of inspiration, in contrast to the sustained negative pressures found during inspiration in the intact, anaesthetized animal (Figs. 4a and b, 5a and b). This change in air flow may be associated with the lower rate of inspiratory movement after vagal section mentioned by Gesell (1940); although in these records it coincides with a lessening in thoracic diameter after about mid-inspiration (Figs. 1b, 4c, 5c). Where this tendency is already present vagotomy subsequently accentuates it (Figs. 5a and c). The combined effects of the slowed rhythm and thoracic diameter change are sufficient to bring about the alteration in air flow.

A B C D

Fig. 4.



A B C D

Fig. 5.

Figs. 4 and 5. Photographic records of the respiratory movements establishing the true time relationships of the thoracic, abdominal and intratracheal pressure records. Vertical alignments from a 1 sec. blinker. T = Thoracic record. A = Abdominal record (both inspiration upwards). I = Intratracheal pressures (inspiratory negative pressure upwards, expiratory positive pressure downwards).

Fig. 4. A. Normal respiration. Thoracic fixation but no inspiratory activity.

B. During administration of 10 p.c. CO_2.

C. Quiet Respiration. Between B and C both vagi were sectioned. Poor thoracic fixation.

D. During administration of 10 p.c. CO_2. Premature thoracic relaxation and marked reflection of diaphragmatic retraction in the thoracic record.

Diaphragmatic retraction coincides with abdominal collapse and maximum positive intratracheal pressure.

Fig. 5. A. Thorax active in quiet respiration.

B. Reflection of diaphragmatic retraction appearing on administration of 10 p.c. CO_2.

C. Quiet respiration. Between B and C both vagi were sectioned.

D. During administration of 10 p.c. CO_2. Similar response to that in Fig. 4 D.

Effect of Increased Respiratory Activity after Vagotomy.

When the vagal reflexes have been removed, an invariable consequence of increased respiratory activity is an accentuation of the premature thoracic relaxation mentioned in the previous section. There is considerable variation from animal to animal in the degree to which this occurs. This is related to the relative diaphragmatic and thoracic activities during the increased respiration. The diaphragmatic retraction may appear only as a notch on the descending (expiratory) limb of the thoracic record (Fig. 1c), or it may appear almost equal to

the previous inspiratory movement (Figs. 1d, 2b, 4d, 5d) producing the appearance of a double thoracic respiratory rhythm.

A nicotine hyperpnoea, from 1 mg. l-nicotine intravenously, contains a number of components not related to the carotid sinus chemireflex drive. Figs. 2b, c and d show how these may cause the thoracic pattern to be markedly distorted. The true thoracic inspiration is becoming less from Fig. 2b to Fig. 2d, where the thoracic record is due mainly to diaphragmatic retraction.

The Diaphragmatic Origin of the Complex Thoracic Pattern.

Although the removal of the vagal reflexes considerably modifies the thoracic pattern by altering the co-ordination of thoracic and diaphragmatic muscular activities, the thoracic respiration can compensate for the absence of the diaphragm. After vagotomy and bilateral phrenotomy the respiratory activity can be raised without producing the characteristic thoracic pattern of the intact animal. Fig. 3 is representative of this series. The abdomen shows poor fixation after inactivation of the diaphragm, either following the thoracic record passively or even collapsing during thoracic expansion. The shifting base-line of the abdominal record in Fig. 3b is due to peristalsis which is easily discernible in these preparations.

Summary of Results.

In the anaesthetized rabbit the patterns of muscular activity from the thorax and abdomen depend on the relative activities from the thorax and diaphragm. The movements of the thoracic cage in part reflect the diaphragmatic actions, particularly at the end of inspiration when diaphragmatic retraction superimposes a deflection on the thoracic record. This deflection becomes more apparent with increased respiration. It is an indicator of diaphragmatic relaxation and enables, together with the intratracheal pressure changes, an analysis of the effects of vagotomy to be made. Vagotomy exaggerates the independence of action of thorax and diaphragm. This becomes particularly apparent if an artificial drive is applied to the centre (Figs. 4 and 5). Electromyography will be necessary for the assessment of the extent and duration of activity in the various muscle groups concerned in the production of the respiratory patterns recorded, and may furnish further evidence of the central separation of respiratory muscle group control.

DISCUSSION.

The changed respiratory muscular patterns after vagotomy show that either the diaphragmatic activity outlasts the thoracic or develops considerably more tension at end of inspiration, causing thoracic indrawing after mid-inspiration. In either case the current concepts of respiratory control offer no ready explanation of vagotomy, altering not only the rhythm but also the co-ordination of the respiratory centre, since removal of the vagi is considered (Pitts, 1946) to allow the respiratory act to continue unaltered save for a slowing in rhythm.

The Organization of the Central Respiratory Mechanism.

The respiratory centre within the medullary reticular formation is responsible for the integrated activity of the respiratory musculature at the abdominal, thoracic and diaphragmatic levels. Under a variety of conditions these levels are capable of independent action (Finley, 1931). The present results suggest that the vagus helps in the co-ordination of this central subdivision. Phylogenetically, the vagus assumes respiratory importance only with the development of diaphragmatic respiration.

The central control of the thoracic musculature is variable in its action from one inspiratory phase to another (Gesell, 1936). The extent to which costal respiration compensates for the removal of diaphragmatic activity is inconstant (Gesell and Moyer, 1931), but is in part related to the age of the experimental animal. In young animals, where vagotomy stops the diaphragm, the thoracic musculature does not adequately compensate until this sensitivity to vagotomy is lost with increasing age (Coombs and Pike, 1930). Similar observations that vagotomy may stop respiration have been made by Koppanyi and Kleitman (1927). Oberholzer *et al.* (1946) have shown a specific pathway over which the inspiratory centre is activated by the vagus in the rabbit. The vagus may then in fact exert a direct tonic influence on inspiration as was suggested in the original Hering and Breuer theory, by Nicholaides (1914) and later by Hammouda and Wilson (*loc. cit.*) and Gesell (1940).

The view that the vagus acts by inhibiting inspiration is due largely to the work of Adrian (*loc. cit.*) and to the majority of recent work (Lumsden, 1923a, b, c, d; Keller, 1931; Stella, 1938; Pitts *et al.*, 1939a, b, c) which by brain stem ablation and stimulation has sought to delimit a medullary dorsal expiratory centre, and a spontaneously active medullary, more ventral, inspiratory centre (apneustic centre) capable of sustained inspiratory activity in the absence of vagal inhibitory influences and a reverberatory inhibitor circuit in the tegmentum of the pons (pneumotaxic centre), which maintains a slowed rhythm in the absence of vagal influences. Others, repeating the intramedullary stimulations of Pitts *et al.*, with the exception of Brookhart (1940), agree that the inspiratory and expiratory centres are discretely situated in the reticular formation of the medulla (Beaton and Magoun, 1941; Magoun and Beaton, 1941; Comroe, 1943).

Monnier (1938) found that pure respiratory effects could seldom be obtained from the ventral and dorso-medial reticular substance; instead stimulation often produced autonomic and somatic motor responses, together with the respiratory changes.

The reticular formation of the brain stem is now known to mediate suppressor and facilitatory functions from a variety of extrapyramidal and subcortical sources (Magoun and Rhines, 1947), so that, as Bach (1948) has pointed out, stimulation of the formation may produce bulbar facilitation or inhibition equally upon vasomotor, respiratory and somatic activity. That the reticular facilitatory mechanism may be in part responsible for apneusis has been suggested by Hoff and Breckenridge (1949) who find that apneusis is not an invariable consequence of vagotomy after low pontine section, and is associated with autonomic imbalance indicating some disturbance of a generalized system.

This view is given considerable support by instances of various procedures such as intracisternal potassium phosphate (Walker *et al.*, 1945), intravenous cyanide and also CO_2 administration which, by virtue of influencing the reticular formation, produce apneustic and decerebrate rigidity phenomena (Ward *et al.*, 1947; Wheatley *et al.*, 1947). The development of apneustic respiration under these circumstances is aided by vagotomy (Walker *et al.*, *loc. cit.*; Taylor, 1930; Barcroft and Margaria, 1932), as it is in bulbar anaemia (Lumsden, 1924).

Central vagal stimulation can "break through" the apneuses in the latter case, causing an acceleration of respiration (Stewart and Pike, 1907). This is similar to the release of rhythmic respiration from apneuses of decerebrate origin by stimulation of the central vagus in the work of Pitts *et al.* (1939b, Fig. 5, G.H.I.; 1939c, Fig. 3A and D), Lumsden (1923) and Henderson and Sweet (1929), who also suspected some affinities of apneusis and decerebrate rigidity.

The correct balance of facilitation and inhibition descending from the medulla permits rhythmic respiration; the medulla is autonomous in this regard but higher areas exert considerable influence on the respiratory rhythm (Sachs, 1911; Ranson and Magoun, 1933; Kabat, 1936; Hess and Muller, 1946). The polypnoea of overheating which originates from the cephalic midbrain (Keller, 1933), continues unaltered in the absence of the vagi in mammals but not in birds (Hiestand and Randall, 1942). The shift in excitable cortex occurring with advancing phylogenetic status is coupled with a parallel movement of areas which influence respiration (Abbie, 1948).

Evidently then, the respiratory rhythm originates from the medullary level but is influenced by a vagal system which extends into the cephalic pons and depends upon the afferent frequency for its effects. Apneusis probably originates outside the respiratory centres and represents a condition of central facilitatory and inhibitory imbalance just as does spasticity or decerebrate rigidity.[1]

Nature of the Vagal Influence on Respiration.

It is apparent from the introductory review and the results of the present investigation that the afferent vagus may not act on the respiration only by way of the expiratory centre, since inspiratory tone is maintained and specific inspiratory excitor vagal pathways are known. Two alternative explanations of the thoracic patterns found present themselves. The vagus either acts more in conjunction with the diaphragm than the thorax, the former possessing a more powerful inherent activity; or else the vagus exerts an excitor effect on the thorax during inspiration up to the time when expiration is triggered. This would produce not only a quickening of rhythm but also maintained tone in the thoracic musculature.

The vagus inhibits reflex motor activity as well as respiration on direct central stimulation (Schweitzer and Wright, 1937) or pulmonary inflation (Johnson and Luckhardt, 1927). There is a period of after discharge in this central inhibition by the vagus which may account for the slowed respiration with intermittent strong stimulation of the vagus, as against the increased rate with similar weaker shocks (Hillenbrand and Boyd, 1936). The prolonged release from apneusis found by Pitts *et al.*, and the persistence of reflex inhibition after the cessation of immediate respiratory effects of vagal stimulation (Schweitzer and Wright, 1937) may then have a common origin.

[1] Addendum. Breckenridge, C. G. *et al.* (1950): Amer. J. Physiol., 162, p. 74, have shown more recently that Myanesin reversibly abolishes apneusis causing phasic respiration to emerge. Since Myanesin is known to block the reticular facilitatory system, these results suggest that apneusis does represent a condition of overfacilitation occluding normal respiration.

The possible projection of the vagus into the corticobulbar suppressor mechanism cannot be overlooked in this regard.

Bailey and Bremer (1938) showed that the afferent vagus projects on to area 13 of the orbital gyrus. This area, together with the cingulate, forms the principle cortical automatic representation. Respiratory, automatic and somatic motor effects can be obtained from these and a variety of other extrapyramidal areas (Smith, 1938; Bailey and Sweet, 1940; Moruzzi, 1940; Delgado and Livingston, 1948; Kaada *et al.*, 1949). The caudate nucleus forms a relay station on the efferent paths from many of these areas (Fig. 6).

It is thus possible for the vagus to exert part of its inhibitory actions from regions not immediately connected with respiratory control. No corresponding mechanism for explaining the excitor effects is known.

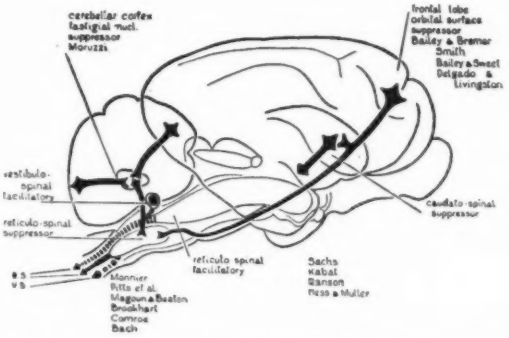


Fig. 6. Reconstruction of the cat's brain[1] showing systems concerned in facilitation (lateral reticulospinal, vestibulo spinal) and suppression (cortico-bulboreticular from pericruciate gyrus, caudatospinal, cerebelloreticular from anterior lobe and paramedian lobules, medial reticulospinal). Workers who have obtained respiratory effects on stimulation are indicated alongside the regions from which these are obtained. The principle respiratory areas of the cortex lie in area 13 on the orbital surface, area 24, and in area 6 adjacent to the sensory-motor cortex. The caudate n. inhibits respiration, the hypothalamic responses are varied. There is a close association within the reticular formation of somatic, autonomic and respiratory effector regions, although the respiratory centres are confined to the region of the obex.

[1] After Lindsley *et al.* 1949.

SUMMARY.

In the rabbit, with light paraldehyde anaesthesia, it is possible to record from the thorax a respiratory pattern which in part reflects the activity of the diaphragm. Diaphragmatic relaxation appears as a deflection (in the inspiratory sense) superimposed on the thoracic respiratory record.

This enables an analysis of the response to vagotomy to be made. Vagotomy causes: (a) Lengthening of the inspiratory and expiratory phases; (b) poor co-ordination of the respiratory activities of the thorax and diaphragm; resulting in a thoracic indrawing after mid-inspiration, the reflection of diaphragmatic retraction appearing on the descending ("expiratory") limb of the thoracic record.

Increased respiratory activity may exaggerate the latter effect. Elimination of the diaphragmatic respiratory activity produces an uncomplicated thoracic pattern.

In discussion, it is pointed out that these results cannot be explained in terms of the current concept that spontaneous respiratory activity is limited to an inspiratory centre not differentiated into diaphragmatic and thoracic parts, vagal or pontine inhibitor paths being necessary for respiratory periodicity. The inherent periodicity of the medullary respiratory centre together with indications of independence at the thoracic and diaphragmatic levels is emphasized.

The present results are taken to indicate that the pulmonary vagal excitor afferents may be more important for the thoracic respiratory mechanism or that the inhibitor afferents are more important in moderating the actions of the diaphragm, permitting balanced diaphragmatic and thoracic activity in the intact animal.

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CANINE ENCEPHALITIS CAUSING A SYNDROME CHARACTERIZED BY TREMOR

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One of the difficulties encountered in attempting to study canine encephalitis is the poorly defined nature of the naturally occurring clinical disorders and the absence of any integrated clinical and pathological studies. The objects of this paper are to define one of the common canine encephalitis syndromes, to provide some information on the chronological development of its clinical signs and to correlate these with the histopathological lesions. The syndrome chosen for study would be referred to in older veterinary literature as "chorea." Since the involuntary movements of human chorea are not rhythmic and the involuntary movements of the canine disease are rhythmic, the term "chorea," as applied to the canine disease, is misleading and should be discarded. The encephalitis syndrome to be described is characterized by muscle tremor, weakness and disorder of muscle tone. It resembles the human tremor-rigidity syndrome or post-encephalitic Parkinsonism in many respects. An important difference between the canine and the human disease is in the severity and nature of the disorder of muscle tone. In the canine disease resistance to passive movement is increased, but the use of the word rigidity would imply a similarity to the human disease, which, at present, does not seem justified. For these reasons, and until more is known of the aetiology, it is suggested that the canine disease be known as the "tremor syndrome."

During this study, being aware of the existence of other encephalitis syndromes in dogs, those cases which did not satisfy our diagnostic criteria were rigorously excluded. As far as possible, therefore, we have deliberately excluded the cerebellar syndrome, the frontal-lobe syndrome and the syndrome of "fits" without focal localizing signs.

Our selection of cases was considerably influenced by the concurrent surgical studies already reported (McGovern *et al.*, 1948) and it is important to realize that the pathology of some of the excluded syndromes may be similar to that of the tremor syndrome. Despite this possibility, it is thought that the canine tremor syndrome, as defined here, represents an identifiable clinical entity which can be used as a corner stone upon which knowledge of the other canine encephalitis syndromes can be built.

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The Incidence of Encephalitis in Dogs Presented to a Veterinary Hospital.

The information in this section of the paper was obtained from the records of the Sydney University Veterinary Hospital (J.D.S.) and from a veterinary practice in a Sydney suburb (M.E.D.). The figures given refer only to dogs.

Although no information was obtained on the aetiology of the tremor syndrome during this study, an attempt was made to correlate the occurrence of the disease with previous or existing distemper infection. For the purpose of diagnosis, distemper was taken to mean any disease in which there had been, or was at the time of presentation, fever, depression, lethargy, loss of appetite, and nasal and ocular discharge. When these basic signs occurred alone or when they were accompanied by vomiting and diarrhoea, or signs of respiratory tract involvement, a diagnosis of distemper was accepted.

Veterinary hospital cases. Among a total of 2,100 dogs presented for examination at the Sydney University Veterinary Hospital during the period from February to September, 1947, there were 120 which clinically manifested some disorder of the nervous system, giving a general incidence for neurological disorders of 5·7 p.c. Of the 120 cases with neurological disorders, 72 (60 p.c.) had, or had had, distemper in one form or another.

Over the same period, there were 237 animals (with and without neurological signs) which were suffering, or had suffered, from some form of distemper. Thus 11·2 p.c. of canine general admissions had clinical evidence of distemper, and of these 30 p.c. showed neurological disorders.

Suburban veterinary practice. In a total of 1,224 dogs presented in the period from July, 1946, to September, 1947, 13·8 p.c. showed clinical evidence of disease of the nervous system, and 15 p.c. had evidence of distemper.

In the group of 170 dogs with various neurological disorders, 50 p.c. had, or had had, clinical evidence of distemper and 33 p.c. of those with clinical distemper showed neurological disorders of various types.

Combining the two series of figures, it would appear that 50–60 p.c. of the neurological disorders in the animals presented are associated with previous or co-existent distemper infection in one form or another. Furthermore, approximately one-third of the dogs which acquire clinically evident distemper infection develop signs of neurological deficit.

It is impossible to provide any definite figures on the incidence of the tremor syndrome in the total group of neurological disorders because many of the case records, which were compiled before the investigation had become crystallized, are inadequate for proper appraisal of the symptomatology. Thirty definite cases of the tremor syndrome were studied clinically and pathologically, and 9 cases were studied clinically, surgically and pathologically. The longest clinical history of any dog in the series studied was 9 months, and the longest period over which any dog was observed constantly was 5 months.

Clinical Features of the Canine Tremor Syndrome.

Age and sex incidence. This condition is a disease of young dogs. Except for one dog which was 11 years old, ages in the present series ranged from 3 months to 3 years, but the majority of animals were aged 1½ years or less. There is no predilection for either sex and no correlation between the age of affected animals and their sex.

Onset. The tremor syndrome in dogs appears to occur in acute and chronic forms, the former being the more common, perhaps because a large proportion of affected animals die or are destroyed in the acute phase of the disease.

In the acute form, the history seldom exceeds two months. The initial complaint in most cases is that the dog has had one of the clinical syndromes commonly diagnosed as distemper. In a number (17 p.c.) of animals there is no history or evidence of distemper. At present it is difficult to assess whether all these latter animals have really not had clinical distemper, or whether reticence or lack of observation on the part of the owner may be a relevant factor; the possibility of subclinical infection must also be considered. In a few cases, usually of the chronic form, up to 2–3 years may elapse between the occurrence of distemper and the commencement of the tremor syndrome. An aetiological relationship between the two conditions is difficult to establish in such cases.

In those cases of the tremor syndrome (83 p.c.) following upon distemper, there appears to be a latent period of a few weeks, usually not more than six weeks and occasionally only a few days, after the initial clinical signs of distemper. This is followed by the development of muscle tremor in one or more limbs or in the head and neck. Although most cases begin with tremor, a few present, as their initial neurological sign, paresis of one or more limbs, which may at once be spastic or else, being flaccid, may become spastic. Somewhat more commonly the syndrome commences with muscular weakness in one or more muscle groups in the limbs, or in the dorsal neck musculature. Thus the syndrome may begin either with tremor, with weakness or with increased resistance to passive movement of the part. These three manifestations of disease represent a symptomatic cycle around which the animal may progress in any direction, beginning at any point.

In those animals having no previous history of distemper, similar observations may be made, and there appears to be no difference in the mode of onset of the neurological disturbances in the two groups although our series of "no clinical distemper" cases is, as yet, too small to permit definite conclusions in this regard.

The chronic form of the disease does not differ in onset from the acute form, in most cases the principle difference being in the clinical course.

Clinical course. At some period, often early in the history of the disease, a change may be noticed in the animal's disposition. From being a docile domestic pet, it may become irritable and resent handling. Or, on the other hand, a previously alert, intelligent dog may become lethargic, lose interest in its surroundings, and even fail to recognize its owner. Changes in disposition are not invariable and are less common in the chronic form of the disease.

In the majority of acute cases, tremor having begun as a more or less localized manifestation (often as clonic movements of isolated muscle fasciculi) quickly spreads to involve the muscles of some or all of the limbs (both flexor and extensor groups of muscles, mainly the former), the muscles of the head and neck, the masseter muscles, the temporal muscles, and occasionally the tongue or the retractor muscles of the eyeball. It has a frequency of 60 to 200 oscillations per minute. At the same time, the dog rapidly loses weight, becomes anorexic and difficult to feed, and muscular weakness may be pronounced, so that the dog is unable to stand. Various pareses may occur in the limbs. Initially the paretic limb is usually flaccid, but it may be spastic or else flaccidity and spasticity may exist in different limbs at the same time, or consecutively in the same limb. The hind limbs seem to be more severely and frequently affected than the fore-limbs.

A common finding, often antedating the onset of paresis, is the progressive development of ataxia, particularly in the hind limbs. Evinced initially by slight swaying of the hind-quarters when walking or trotting, the animal soon finds difficulty in going up steps or lifting its hind legs over obstacles. Prehension may also become ataxic. Eventually ataxia is profound and the animal cannot stand or walk. An asymmetric head posture may be seen and, at times, the animal walks in a circle, following the direction of the deviated head. In some cases, this is associated with a horizontal or rotary nystagmus and head nystagmus may also occur.

The reflexes may be disturbed in various ways. In nearly all cases the placing reactions disappear on one or both sides, the fore-limbs being more frequently affected than the hind-limbs. The landing reflexes are often impaired. Both the placing reactions and the landing reflexes are absent in severely paraplegic cases. The withdrawal reflexes are nearly always present and, when absent, the hind-limbs are more often affected than the forelimbs. The tendon reflexes may be increased even in the presence of flaccid paresis.

The pupillary reactions are disturbed in a number of animals, loss of the light reflexes on one or both sides being the commonest observation in this regard. Anisocoria has also been noted on a number of occasions, while in other animals both pupils are widely dilated and unreactive. In these latter cases, the eye preservation reflexes are absent and the animal runs into objects, apparently blind. No abnormalities of the fundus have been detected ophthalmoscopically. No strabismus has been seen. Evidence of disturbance of trigeminal and facial function is provided by absence of one or both corneal reflexes, trismus, tremor of the eyelids or tremor of the buccinator muscles and proper levators of the nose. Occasionally tremor of the tongue is noted. A common finding, in both acute and chronic cases, is excessive salivation. A jerky "cogwheel" type of inspiratory rhythm may be seen.

Some animals have generalized motor seizures without loss of consciousness, that are ushered in by extensor spasms of the limbs and opisthotonus, at the onset, during the disease or in the terminal phases. They appear to be precipitated by handling or by a strange environment and the extensor phase is followed by paddling movements of the limbs and champing of the jaws, which lasts some minutes. Other animals exhibit localized motor seizures which are confined to the head. These seizures may occur without loss of postural control and are characterized by excessive salivation, excessive blinking, clonic movements of the muscles of the head and champing of the jaws. Both types of seizure often occur in repetitive episodes.

In animals with the acute form of the disease, progression is rapid and the animal may die within a few days or weeks of the onset of the disease. In the chronic form of the disease, disturbances of function are similar in type and distribution, but in this case the disease process appears to progress to a certain point and then becomes stabilized, the symptomatology remaining fixed, so that the distribution of tremor, weakness and resistance to passive movement may remain constant for a period of months. Even in these cases, however, there may be episodic exacerbations at long intervals with concomitant alterations in the symptomatology. In these animals it is common to find isolated tremors of one limb or even of one muscle, or spasticity of the head and neck, or of one limb. Marked paresis is unusual, and ataxia is only slight or moderate in degree. Cranial nerve involvement in these animals appears to be less frequent clinically than in the acute form and anorexia and marked loss of weight do not occur. Localized wasting in the affected muscles may be seen and it remains restricted to the affected parts. In long standing cases, abnormal wearing of the toe pads and nails is evident, and in some cases localized alopecia and abnormal callus formation may be seen on the dorsal surfaces of the terminal phalanges or on the plantar aspects of the tarsal joints. "Fits" do not appear to occur in the chronic form of the disease.

In brief, in both the acute and chronic forms, tremor is the most frequent finding, less frequent is muscular weakness and still less frequent is the occurrence of spasticity which may be present in slight degree only in the most acute cases, but is rather more prominent in the chronic form of the disease. When distinctly present, this increased resistance to passive movement frequently has a jerky "cogwheel" character.

Discussion of the Clinical Findings.

As can be seen from the above description, individual cases may show a wide variety of clinical signs indicative of greater or lesser degrees of nervous involvement but all cases showed two or more of the basic disturbances simul-

taneously or successively. The canine tremor syndrome is not a new disease and many of the clinical signs described here can be found in the reports of the clinical features of so-called "nervous distemper" by Dunkin and Laidlaw, 1926; Perdrau and Pugh, 1930; Marinesco *et al.*, 1933; Hurst *et al.*, 1943, and others. After reading the reports of these authors there seems little doubt that examples of encephalitis causing the tremor syndrome have been seen. There seems equally little doubt that other cases of encephalitis showed clinical signs not characteristic of the tremor syndrome.

Little is known of the aetiology of encephalitis in dogs, and this study has provided no information on the aetiology of the tremor syndrome. We do not know whether the tremor syndrome represents an aetiological entity or whether it may result from a variety of insults to the central nervous system. We also do not know whether the pathological processes described below invariably give rise to the tremor syndrome or whether other encephalitic syndromes are occasionally or frequently associated with the same pathological picture.

Pathology of the Canine Tremor Syndrome.

Material and Methods.

The brains of 25 dogs manifesting this syndrome in the acute phase, five in the chronic phase, and a brain biopsy from area 6 of one of these animals in the chronic phase have been examined.

After fixation in Heidenhain's Susa in some cases, and in 10 p.c. formol-saline in others, sections were taken from the cerebral cortex, including areas 4, 6 and 8, the basal ganglia, midbrain, hypothalamus, pons, cerebellum, medulla oblongata and spinal cord. Frozen sections of formalin-fixed material were stained by various modification of Rio-Hortega's silver carbonate methods for microglia, oligodendroglia and astrocytes. Paraffin sections were stained by Mayer's haemalum and eosin, Mallory's phosphotungstic acid haemotoxylin, Nissl's stain, Weil's myelin stain and Foot's modification of Roger's stain for neurofibrils.

Changes in the Acute Phase.

The acute reaction is essentially a demyelinating or necrotising lesion, focally distributed, and ranging in severity from an area of patchy degeneration to an area of colliquative necrosis. This process has been observed in various stages of progression in the same brain on all occasions.

The earliest pathological manifestation of the disease appears to be the formation of an area of microglial infiltration; and as the lesion progresses, myelin sheaths are destroyed and compound granular corpuscles become recognizable in all phases of development. At this stage, the lesion may form an actual softening, but more commonly some of the original framework of the affected area remains. Glial cells proliferate and gemästetezellen become recognizable. The compound granular corpuscles then gradually disappear, leaving a vacuolated appearance which persists despite an intense glial reaction in which very large fibrous astrocytes are the chief component. Oligodendroglial proliferation is not marked. Areas of less severe involvement, where demyelination is minimal, are also repaired by fibrous astrocytes. Inflammatory cells of haemic origin, mainly lymphocytes and plasma cells, may be recognized in these areas, but they are very few in number unless there is some associated perivascular inflammation.

This focal inflammation which may be observed in various stages of development in any one brain, has no vascular orientation. However, in certain areas, particularly in the grey matter of the cerebrum, the vessels within the lesion exhibit a characteristic reaction. This consists of swelling and proliferation of the endothelial lining of the capillaries and arterioles. Although varying degrees of vascular occlusion are thus produced, no thrombosis has been observed. In more advanced lesions infiltration of the walls and around the vessels by lymphocytes and plasma cells may occur. This type of vascular change is less frequently seen in the brain stem or cerebellum.

Perivascular cuffing by lymphocytes and plasma cells is found in the brains of dogs with the longer histories of the tremor syndrome. It has not been observed in the earliest demyelinating lesions, but in later stages it may be observed both alone or within foci of acute demyelination in the same brain. In Case 18, there are foci of acute demyelination unassociated with vascular changes, other similar foci with these vascular changes, and throughout the brain there are numerous vessels with heavy infiltrations in their walls and surrounding them. This animal had exhibited the tremor syndrome for two months, a longer period than any of the other animals in the acute phase.

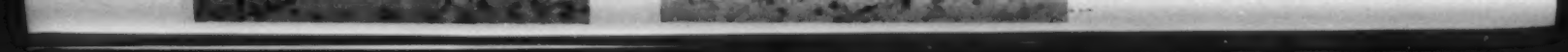
Sites of Inflammatory Reactions.

The acute focal demyelinating process may be observed in any region of the brain or, less often, of the spinal cord. Such foci are constantly present in the cerebellar white matter and in the roof of the fourth ventricle. Sometimes lesions in the folia are sufficiently extensive as to involve the granular, and occasionally the Purkinje cell layers; but only exceptionally is the molecular layer involved. (This is in marked contrast to the descriptions of the usual findings in cases of "distemper encephalomyelitis," in which the molecular layer of the cerebellum is intensely affected; e.g. Marinesco *et al.*, 1933). While no part of the cerebellum is immune from this type of inflammation, certain areas of this organ are selected in a manner related to their anatomical arrangement. Extension of the lesions present in the roof of the fourth ventricle frequently affects the nuclei fastigii, portions of the dentate nuclei and the flocculonodular lobules. In addition, extension occurs into the posterior portion of the palaeocerebellum in the subependymal regions, through the angles formed by this structure with the roof of the fourth ventricle; these various extensions of the inflammation occur where there is no molecular layer. The molecular layer appears resistant to this type of inflammation, and commonly one finds subpial inflammation in the depths of sulci where there is no subpial molecular layer. Moreover, in such cases the overlying pia seldom shows much inflammation.

Other common sites of involvement are the brain stem, the cerebral cortex, and the caudate nucleus in that order of frequency. In the brain stem, subpial demyelination may be patchy, or it may encircle the brain stem completely. Such a lesion commonly involves the subpial portion of the cerebellar peduncles and the dorsolateral aspect of the medulla oblongata near the restiform bodies and vestibular nuclei. The caudate nucleus is often affected to various degrees by the inflammatory process, the lentiform nucleus much less frequently. Other affected sites observed in this series are the hypothalamus, the periventricular nuclei and the grey matter of the spinal cord.

The acute perivascular infiltrations are variable in their occurrence and distribution. They more often affect the white matter than the grey matter, and at times the grey matter of the cerebrum is quite unaffected by any inflammatory lesion, while extensive perivascular inflammation involves the subjacent white matter. The cerebellum is sometimes affected by this type of lesion.

Petechial haemorrhages are of frequent occurrence in various sites in the brain and cord, but their inconstancy and their recent appearance suggests that in some cases they may be agonal. In this regard, it might be noted that the majority of the dogs used in this study



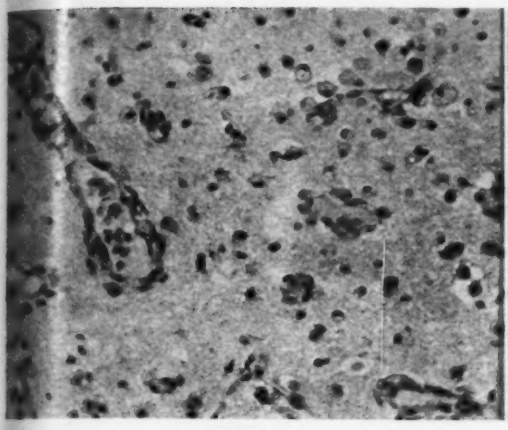


Fig. 1.

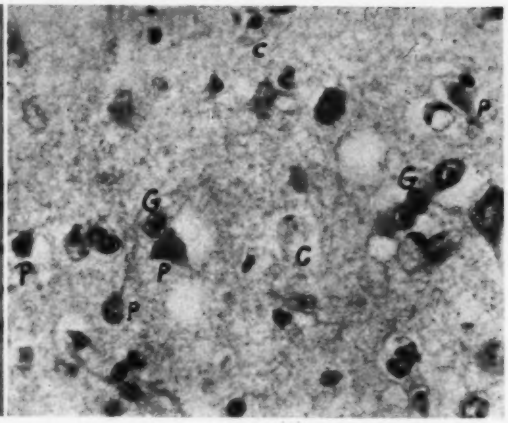
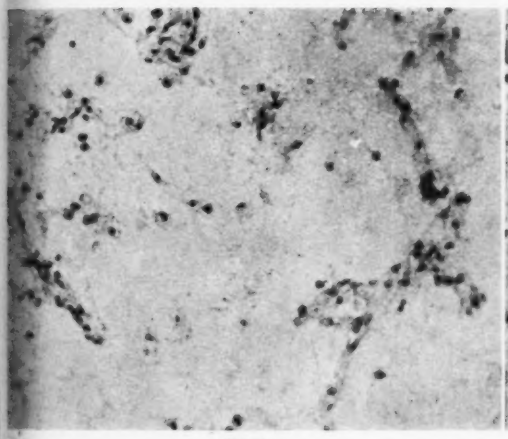


Fig. 2.



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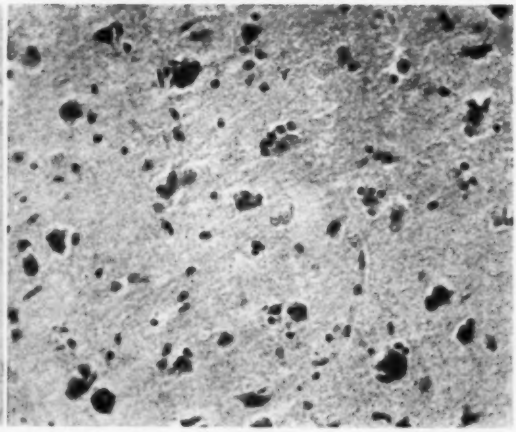


Fig. 4.

Fig. 1. Case 6. Acute. An area of acute inflammation in the cerebral cortex. The vessels are very prominent. Compound granular corpuscles in all stages of development are present. Neurones are degenerate and not recognisable as such in the photomicrograph.
Haematoxylin and Eosin. × 200.

Fig. 2. Case 14. Acute. An area of acute inflammation in the cerebral cortex at a higher magnification, showing degenerate pyramidal cells (P), compound granular corpuscles (C), and glial cells containing nuclear inclusions (G).
Haematoxylin and Eosin. × 400.

Fig. 3. Case 8. Acute. A softened area in the cerebral grey matter. Only vessels and a few small compound granular corpuscles remain.
Haematoxylin and Eosin. × 200.

Fig. 4. Case 18. Acute. Area 6 of the cerebral cortex showing neuronophagia.
Haematoxylin and Eosin. × 200.

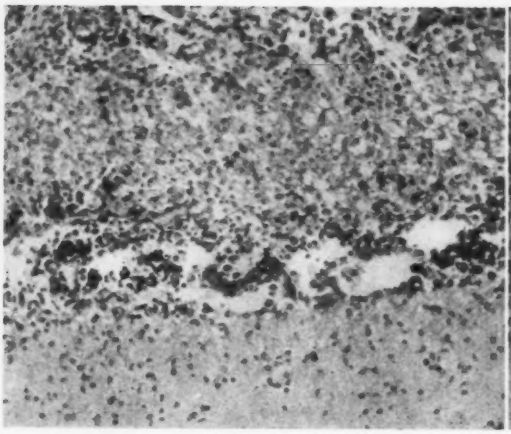


Fig. 5.

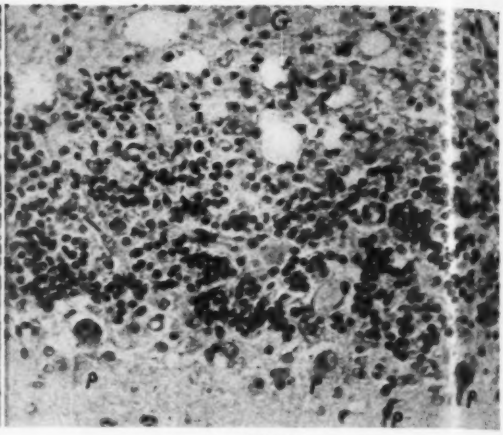
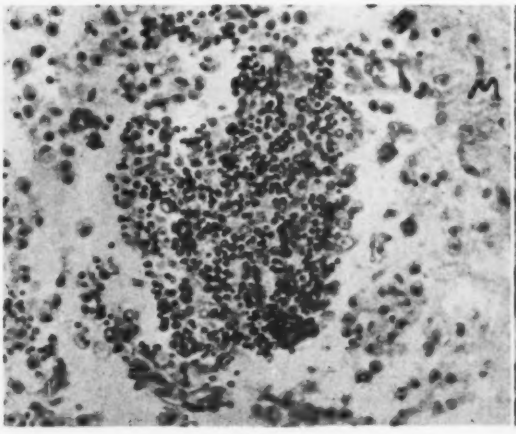


Fig. 6.



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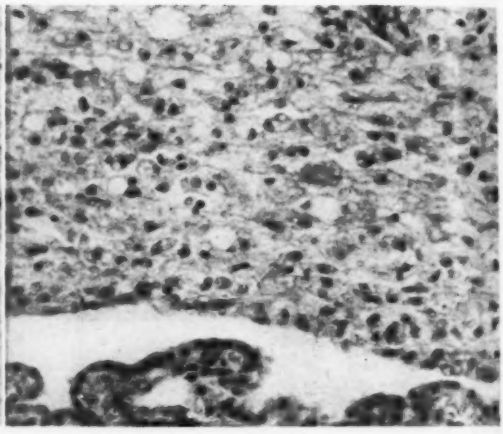

Fig. 8.

Fig. 5. Case 6. Acute. Demyelinating lesion in the white matter of the cerebellum involving the granular layers. Compound granular corpuscles are numerous.

Haematoxylin and Eosin. × 100.

Fig. 6. Case 8. Acute. Demyelinating lesion similar to that in Fig. 5, in which the compound granular corpuscles have mostly disappeared, leaving a vacuolated appearance. Glial cells are proliferating and gemästetezellen (G) are recognizable. Purkinje cells (P) are degenerate and shrunken.

Haematoxylin and Eosin. × 200.

Fig. 7. Case 14. Acute. A demyelinating lesion in the cerebellum has progressed to colliquative necrosis. The granular layer is involved, but not the molecular layer (M). Purkinje cells cannot be recognized.

Haematoxylin and Eosin. × 200.

Fig. 8. Case 6. Acute. Roof of the fourth ventricle in the lateral recess, showing acute demyelination.

Haematoxylin and Eosin. × 200.

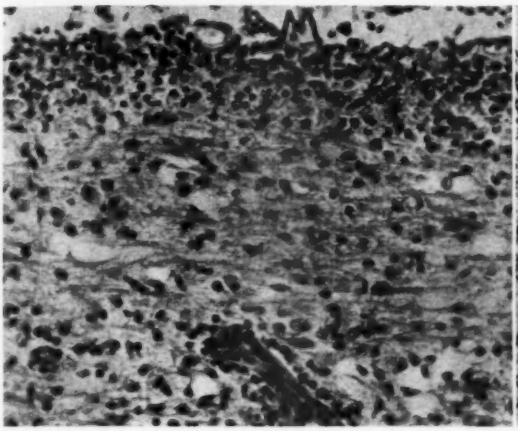

Fig. 9.

Fig. 10.

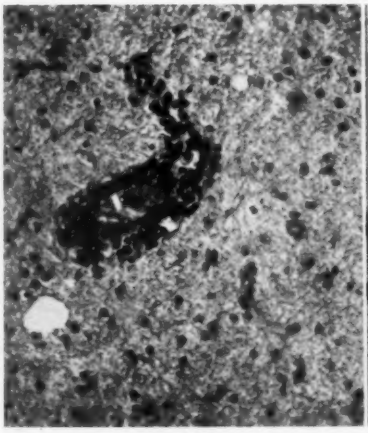

Fig. 11.

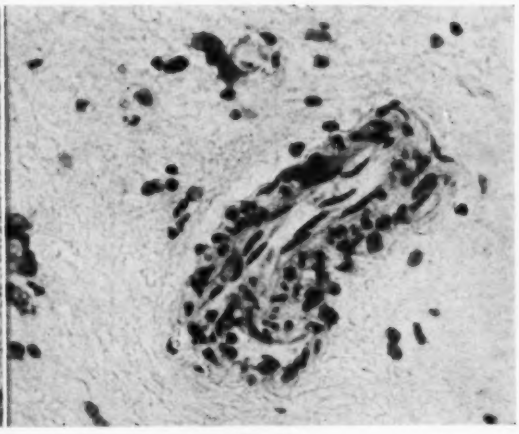

Fig. 12.

Fig. 9. Case 16. Acute. Acute inflammation in the optic chiasma. M indicates the meningeal surface. The inflammatory cells are glial, compound granular corpuscles, and a few mononuclear haemic cells. This type of inflammation is the commonest reaction observed in the subpial regions of the brain stem. A vessel with some perivascular cuffing is also present. Haematoxylin and Eosin. × 200.

Fig. 10. Case 8. Acute. Subependymal inflammation involving the caudate nucleus. The inflammatory reaction consists of glial proliferation and also lymphocytes and plasma cells. The subjacent vessels show perivascular inflammation.
Haematoxylin and Eosin. × 100.

Fig. 11. Case 18. Acute. Perivascular collections of lymphocytes and plasma cells.
Haematoxylin and Eosin. × 200.

Fig. 12. Case 21. Chronic. A small vessel just below the inferior colliculus in which nuclear remnants of inflammatory cells still remain. Nissl stain. × 400.

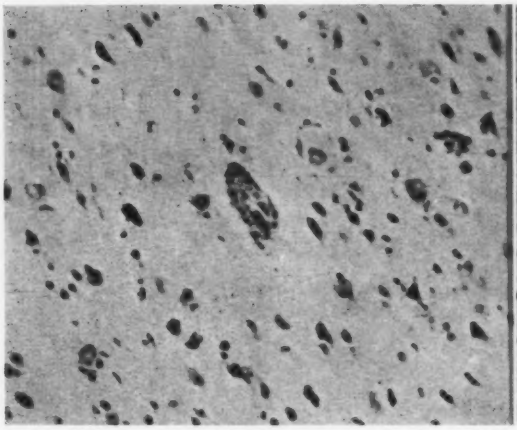

Fig. 13.

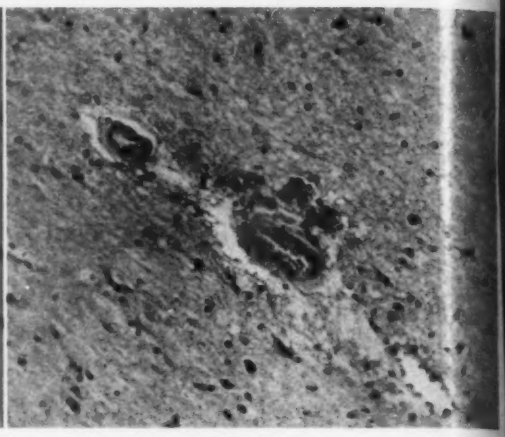

Fig. 14.

Fig. 13. Case 20. A small vessel with thickened walls in the cortex. Neurones surrounding this are degenerating and dropping out. Nissl stain. × 200.

Fig. 14. Case 21. Thickened vessels in the mid brain from which most of the appearances of inflammation have disappeared, leaving an appearance resembling that of arteriosclerosis. Haematoxylin and Eosin. × 200.

were destroyed by intravenous or intracardiac injection of pentothal sodium, saturated magnesium sulphate solution or ether, although others died as a result of the disease.

The optic tracts and chiasma are often affected both by the demyelinating and the perivascular inflammatory processes. Such inflammation is maximal at the circumference of the tracts, and fades off towards the centre.

Neuronal Changes.

Neuronal degeneration is commonly seen in relation to the inflammatory foci described above. The smaller pyramidal cells in areas 4, 6 and 8, but particularly in area 6, of the cerebral cortex appear to be very susceptible to degenerative change, while the large Betz cells of area 4 are seldom affected to any marked degree. The other smaller cortical neurones are also resistant. In acute lesions, the smaller pyramidal cells may be represented only by an occasional eosinophilic ghost cell, with or without a pyknotic nucleus. However, even in the absence of florid inflammatory changes, increase in glial cells, satellitosis and neuronophagia are frequently seen in relation to neurones of the cerebral cortex, caudate nucleus and the midbrain. The changes in the pyramidal cells of the cerebral cortex consist of pyknosis and eccentricity of the nucleus, and the development of corkscrew appearance of the axones.

In an occasional case, pathological changes have been observed in hippocampal structures, consisting in eosinophilic degeneration of neurones with pyknosis and eccentricity of nuclei.

The periventricular nuclei of the diencephalon are often involved in the inflammatory processes, but seldom is any significant change observed in the neurones of the lentiform nucleus, the substantia nigra, the pontine nuclei or the medullary nuclei (apart from the vestibular nuclei), except in those cases where an acute inflammatory focus is situated in their vicinity.

Some slight degenerate changes, but no inflammatory involvement, may be seen in the olive, the neo-olive showing slightly more toxic degeneration than the palaeo-olive. The possibility exists that these appearances are due to the methods of fixation and dehydration of the tissues.

The Purkinje cells of the cerebellum, particularly in relation to foci of arboreal degeneration of the type described above, show patchy degeneration, and in some areas there are many shrunken and distorted cellular remnants. The nuclei fastigii and dentate nuclei, however, are frequently involved by the inflammatory changes in the roof of the fourth ventricle.

Spinal Cord.

In the spinal cord, inflammatory processes of the above types are seen in various portions of the central grey matter, but only in the more severely affected animals. Otherwise, an increase in microglia is the only common observation in the cord. The cord lesions being relatively of minor degree as are the meningeal lesions, the term encephalitis has been used rather than encephalomyelitis or meningoencephalitis.

Ependymal Lesions.

Patchy subependymal inflammation is a common lesion, particularly in that portion of the intraventricular ependyma overlying the caudate nucleus. It is characterized by subependymal proliferation of glial cells, interspersed amongst which are varying numbers of lymphocytes and plasma cells. Occasionally there is a small subependymal haemorrhage. In the roof of the fourth ventricle, the subependymal inflammation is more often of the acute demyelinating type described earlier, but it may also involve the nuclei in both roof and floor of the fourth ventricle.

Meningeal Lesions.

Congestion of the meningeal vessels and small subarachnoid extravasations of red blood cells are frequent observations. In places, there is patchy infiltration of the leptomeninges by lymphocytes and plasma cells, but seldom of marked degree. Vessels entering the brain may carry them with a cuff of inflammatory cells. Macroscopically, the dura is usually thickened and vascular, and small petechial haemorrhages therein are commonly seen both at operation and at autopsy.

Myelin Studies.

There appears to be no specific tract degeneration within the brain or spinal cord. There is, however, spotty demyelination demonstrable in those cases with lesions of the cerebellar peduncles, the arbor vitae of the cerebellum and in the cortico-spinal tracts of those cases with severe cortical degeneration. Similar lesions may be seen in the subcortical "U" fibres beneath areas 4 and 6. The absence of marked spinal cord lesions in these cases contrasts with the findings in cases of so-called "distemper encephalomyelitis" (Perdrau and Pugh, 1930; Marinesco *et al.*, 1933; Hurst *et al.*, 1943). No studies by the Marchi method were attempted, so that small groups of degenerating myelin sheaths have not been detected.

Inclusion Bodies.

In the areas of acute inflammation, nuclear inclusions are often present. They vary in number from one to four within a single nucleus, a solitary intranuclear body being the more common finding. They appear to be homogeneous in structure and are intensely acidophilic. They are spherical or rhomboidal in shape. They have also been noted, under similar circumstances, by Marinesco *et al.* (1933), Hurst (1943) and others. These inclusion bodies are

present in our cases only in the nuclei of glial cells. In some instances the cytoplasm has disappeared leaving naked nuclei, making identification of the cells impossible; but in other cases, cells containing nuclear inclusions are recognizable as protoplasmic astrocytes. The nuclei containing these bodies are larger than usual, and display margination of their chromatin, producing a superficial resemblance to small neurones; such resemblance is more marked in those nuclei which possess nucleoli.

In no case can nuclear inclusions be detected in the cells of the perivascular infiltrations nor in the cells of the ependyma. In a few instances, however, they are present in the cytoplasm of compound granular corpuscles. No such bodies can be detected in neurones, but this may be due to pyknosis of the nuclei of degenerating neurones preventing their identification. In the usual forms of "distemper encephalitis" inclusion bodies have been described in neurones in various sites (e.g. Marinesco *et al.*, 1933; Hurst, 1943).

The number of intranuclear bodies present has no correlation with the severity of the lesion. They are more numerous in early lesions, and are rendered more prominent by the use of Heidenhain's Susa for fixation of the tissue. They have been found in the spinal cord in one case only.

Of the twenty-five acute cases examined, nuclear inclusion bodies are demonstrable in various sites in twenty three.

Changes in the Chronic Phase.

The brains of five dogs in the chronic phase, together with a biopsy specimen of area 6 of the premotor cerebral cortex of one of them, have been examined. In none of these cases was the disease fatal, and so the lesions are less severe than in the acute phase. However, the character and distribution of the lesions are consistent with those found in the acute phase. In those brains (three) from which a portion had been removed at operation, there is still active repair in progress at the operation site.

Vascular Changes.

While no acute demyelinating or necrotizing lesion remains, there is still in some brains an occasional vessel with a cuff of lymphocytes; apart from these, throughout the brains, in areas where acute lesions usually occur, the vessels show the effects of past inflammation. This is a characteristic thickening, partly cellular and partly fibrinoid. The cellular element consists in part of inflammatory cells and degenerating nuclei which are presumed to be remnants of a more acute inflammatory infiltration. As a result of these changes, the lumina of these vessels are reduced in diameter and certain of the pathological changes amongst the nerve cells appear to be dependent upon this.

Cerebral Cortex.

Excluding the site of operation in those animals subjected to surgery, the most prominent feature in all cases in the chronic phase, is degeneration of the smaller pyramidal cells in the motor and premotor areas of the cortex. These degenerations are focally situated and consist of pyknosis and eccentricity of nuclei, thickening of axones, and dropping out of neurones. Around the thickened vessels, there are fewer neurones than should be present and many that remain are mere ghost cells. In some foci, disorientation of the neuronal axes is seen. The large Betz cells of area 4 are little affected.

Basal Ganglia.

Some vessels show the changes already described, but to a lesser degree, and the neuronal degeneration which is also minor, is neither extensive nor prominent.

Diencephalon.

An occasional thickened vessel in the thalamus can be found. In one brain, however, there is quite extensive residual inflammation consisting of microglial foci, some perivascular cuffing and quite marked neuronal degeneration.

Midbrain.

An area of demyelination between the medial borders of the substantia nigra is present in one brain. In the other brains some vascular thickening but not sufficient neuronal damage of an easily assessable degree can be observed. Increase in glial cells in the subpial zones is present, but no obvious demyelination.

Medulla.

There is slight fibroglial increase in the floor of the fourth ventricle together with occasional thickened vessels, but in only one brain is a nuclear mass involved. The vestibular nucleus here shows eosinophilic degeneration of the cells.

Cerebellum.

Glial cell increase in the subependymal and subpial portions of the arbor vitae is present but not to the same extent as is found in the dogs with acute lesions. Nor are the roof nuclei involved. Thickened vessels are present in the cerebellum, but there is little damage to the surrounding tissue. Purkinje cells do not show recognizable diminution in numbers.

Spinal Cord.

There are no obvious cord lesions in the chronic cases.

Myelin Studies.

In those animals from which a portion of the cerebral cortex had been removed surgically, there is a corresponding myelin deficit in the pyramidal tracts. There are few obvious foci of demyelination relating to the other lesions and those that are demonstrable, are very small perivascular lesions.

Inclusion Bodies.

Inclusion bodies have not been observed in the chronic cases.

To summarize, the most important and the only constant neuronal lesion in dogs in the chronic phase of the encephalitis under investigation is in the cerebral cortex. Other lesions are scattered throughout the brain but are much less extensive.

Discussion of the Histological Changes.

Earlier writers (Perdrau and Pugh, 1930; Marinesco *et al.*, 1933; Hurst *et al.*, 1943) have described lesions in "nervous distemper" which resemble, in some respects, those described in the present study. However, they emphasize the perivascular distribution of the demyelinating process. Perdrau and Pugh state that the lesions are often orientated about a group of vessels; Weston Hurst *et al.* support this observation. The interpretation of the present observations is that there are two distinct pathological processes which may occur independently in the same brain, or concurrently in a single lesion.

The earliest lesion, and the most frequently observed, is a demyelinating process in which myelin sheaths are disrupted, and in which glial cells and neurones degenerate. This process may progress to colliquative necrosis, or, becoming halted, the lesion may be replaced by reactive gliosis. The cells present in this type of reaction are glial and microglial. Cells of haemic origin are uncommon. In the white matter of the cerebellum, the subpial zones of the brain-stem and grey matter of the cerebral hemispheres, this type of pathological process is the most common. There is no perivascular orientation in this type of lesion, but vessels within the lesion, particularly in the cerebral cortex, may show swelling and proliferation of endothelium. Unless destruction is very marked, neurofibrils traversing the area are quite likely to remain intact.

Perivascular infiltration is regarded in this study as a further process. Its inconstancy and its absence from the earliest demyelinating lesions in the cerebellum and brainstem support this view. It appears to occur at a later stage than the demyelinating inflammatory reaction, and though it may occur concurrently with the latter, it has not been found in the brains of the animals with the shortest histories.

In the chronic cases, in areas of residual inflammation and in areas in which the inflammation has subsided, thickened vessels remain. The thickening is partly cellular and partly fibrinoid. The nuclei in the vessell wall are pyknotic and probably result in part from the endothelial proliferation described earlier, and in part from the lymphocytic cuffing. This feature is prominent in all the cases exhibiting the chronic form of the disease, and is present also in case 18 which, pathologically, is midway between the acute and the chronic phase. Vascular thickenings have been observed in the brains of humans affected by the tremor-rigidity syndrome and although thought by many to be arteriosclerotic, the interpretation of Benda and Cobb (1942) is that some of these are residua of inflammatory lesions.

A constant observation, not stressed by earlier writers, is the presence of lesions in the cerebellum of acute cases. In all cases, the archicerebellum and the fastigial nuclei are affected. The palaeocerebellum is affected to different degrees in both the anterior and posterior portions in different cases. The neocerebellum shows similar lesions mainly in the superior vermis and ansiform lobes; the dentate nuclei and their projections are seldom severely affected. As previously mentioned, the vestibular nuclei may be involved.

Even where there is no inflammatory reaction, the smaller pyramidal cells of the cerebral cortex, particularly in area 6, undergo degenerative changes. In the chronic cases, this is probably aggravated by the partial vascular occlusion due to the thickening of small arterioles.

SUMMARY.

A study has been made of an encephalitis in dogs characterized clinically by tremor and resembling the human tremor-rigidity syndrome in many respects. This encephalitis has been described from the clinical and pathological aspects. It is frequently associated with previous clinical distemper. The pathological changes are those of a meningo-encephalo-myelitis in which nuclear inclusions are present within the glial cells.

The lesions constantly present in acute cases are those of focal demyelination in the cerebellum and neuronal degeneration in the motor and premotor cortex. Other characteristic but not constant lesions are subpial demyelination of the brainstem and focal demyelination in any portion of the brain.

Residual lesions in the brains of dogs that have survived the encephalitic process and in which the clinical signs are static, consist of thickening of the smaller arterioles, degeneration of the pyramidal cells of the premotor and motor cortex and scattered minor residua throughout the brain.

Acknowledgments. Thanks are due to members of the School of Veterinary Science and Department of Surgery, who facilitated this combined study. The laborious histological work was performed by Mr. Bruce Munro of the Department of Pathology. The extensive photographic studies were made by Mr. S. Woodward-Smith of the Department of Medical Artistry.

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OESTROGENIC EFFECTS OF SUBTERRANEAN CLOVER (*T. SUBTERRANEUM L.* VAR. *DWALGANUP*): MAMMARY DEVELOPMENT IN THE CASTRATE MALE GUINEA-PIG[1]

by JUNE EAST

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In 1944 Bennetts first investigated a serious breeding problem affecting sheep maintained on dominant early subterranean clover pastures in Western Australia. Later Bennetts, Underwood, and Shier (1946) reported three characteristic clinical manifestations presented by the ewes, namely dystocia, uterine prolapse, and infertility. They also noted the frequent appearance in these affected animals of a cystic glandular hyperplasia of the uterine epithelium, and considered this condition to be indicative of some type of hormonal disturbance. In the same paper the authors drew attention to the occurrence of mammary enlargement in both unbred females and castrate males (wethers).

Numerous cases of udder development and milk secretion in virgin and unbred ewes, particularly during the spring and early summer months, were found on many properties possessing dominant subterranean clover stands. The microscopic picture was that of a normal functioning mammary gland with full alveolar development, while analysis showed the secretion to be similar to colostrum. Lactation in wethers was continually reported during the spring of 1942–3, in one instance about 8 p.c. of the total number of wethers carried showing evidence of lactation. The proportions of the teats corresponded to those of ewes in full lactation, the nipples being two and a half inches long and two inches in diameter at the base in a particular animal examined. The amount of secretion obtainable from a single teat varied from 2 to 18 ml. and was normal in constitution. Histologically the glandular secretory tissue appeared to arise from a single layer of columnar cells lining the teat wall.

These findings were further substantiated by Bennetts (1946), who produced both mammary development and milk secretion in two Merino wethers, by intramuscular injection of 1,200 mg. stilboestrol over a four week experimental period. Such results, and the demonstration of squamous metaplasia in the accessory reproductive organs of the castrate male sheep maintained on potent herbage, provided strong presumptive evidence that the "affected" clover contained some principle (or principles) which was either an oestrogen or an oestrogen precursor.

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As cystic endometrium had been successfully produced in female guinea-pigs after ingestion of fresh green air-dried, or artificially dried clover (Bennetts *et al.*, 1946; Curnow, Robinson and Underwood, 1948), another series of experiments was designed using the castrate male animal. Here again a characteristic sequence of epithelial changes, comparable to that obtained in wethers, was seen (East, Underwood and Bennetts, 1949). Noticeable also were the increased dimensions of the mammae, although milk secretion did not occur. Actually these latter investigations were implemented to determine whether androgens would control or eliminate the hypertrophic sequelae of clover feeding or oestrogen injection, the nipple measurements being made simply to provide possible quantitative estimates of treatment differences. However, they proved to be such statistically sound criteria of hormonal effects, that similar estimations were made in conjunction with a further extension of the work to elucidate the reaction of the adrenal and pituitary glands to oestrogenic stimuli.

This report records the results of experiments devised to investigate more fully the response of the castrate male guinea-pig to a known potent clover diet. The emphasis is placed here on mammary reaction, and the desirability of developing such a nipple length test as an assay procedure by future trials planned specifically for this purpose. Comments on the adrenal and pituitary are reserved for a separate paper, and similarly only brief mention is made of the microscopic picture presented by the accessory reproductive organs, this being substantially the same as that detailed in the earlier publication (East *et al.*, 1949).

In the following survey stress has been laid on factors effecting nipple development alone, and the controversy regarding the direct or indirect action of oestrogens on the mammary gland has been included only insofar as it is consistent with this purpose.

Distinction has been made between the phrases "mammary development" and "nipple growth." The former is intended to include both duct and lobule-alveolar extension, while the latter refers exclusively to the teat as distinct from the corpus mammae. Enquiry into process of lactation has not been attempted.

In 1911 Aschner and Gregoriu observed that aqueous extracts of the placenta produced growth of the mammary glands in male and female guinea-pigs, and as early as 1925 Loewe demonstrated nipple enlargements in animals receiving crude oestrogenic hormone preparations. Zondek (1926) also made reference to this fact, although negative results were attested by Laquer, de Jongh and Tausk (1927) following the injection of small doses of menformon (Organon) into castrate male guinea-pigs for as long as six weeks. Further work proved more successful, proliferation of the glandular system and growth of the teats equal to that of pregnancy being secured (Laquer, Borchardt, Dingemanse and de Jongh, 1928). In the same year Steinach, Dohrn, Schoeller, Hohlweg and Faure, injecting aqueous solutions of oestrogenic hormone in doses of 25 m.u. per day for three weeks caused the nipples of castrate male guinea-pigs to increase from 1 mm. to 7 mm. in length, these dimensions approaching those of normal seventeen week old female animals.

Smelser (1933) also employed guinea-pig nipple length as a measure of sensitivity to oestrogenic substances. Daily injections of as little as 0·05 r.u. of oestrin over a period of twenty days gave minimal response, while with 0·125 r.u. and 0·25 r.u. increases of 30 p.c. and

70 p.c. respectively over the normal were obtained. He then injected comparable groups of normal and gonadectomized animals of both sexes with oestrin alone, and combined with 7 b.u. of testis hormone. The final teat lengths were the same in both cases and, doubtless influenced by his close association with Moore and Price, Smelser considered the result proof that the testis hormone was not a deterrent to the glandular responses elicited by the oestrin, i.e. he refuted the "antagonistic" theory of sex hormone action. In this context it seems reasonable to assume that relative dosage levels were of some importance, Jadassohn, Fierz-David and Pfanner (1942) using 5,000 μg. testosterone to nullify the effect of 1 μg. oestrone on the guinea-pig nipple. In 1934 Turner and Gomez injected adult male guinea-pigs with increasing amounts of theelin in oil giving 1, 2, 4, 8, 12, 16 and 20 r.u. daily for forty days. They found that those animals receiving 1 r.u. theelin each day showed least nipple responses, whilst those getting 2 and 4 units grew more rapidly. The guinea-pigs injected with 8 or more units appeared to grow quicker during the first twenty days but showed less difference at the end of the experimental period, and the pigs receiving 16–20 r.u. for the entire time presented teats of the greatest length. Lewis and Turner (1942) using diethylstilboestrol found that doses of 0·01 μg. caused 30 p.c. increases in nipple length, but that maximal dimensions were attained after administration of 0·10 μg. (153 p.c.). However, larger amounts of 0·5, 10·0 and 50 μg. had no greater effect on the organs.

Literature concerning the results of androgens alone on the nipple is in many cases conflicting. Work done by Bottomley and Folley (1938) suggests that there is a correlation between chemical constitution and the power of these hormones to stimulate teat growth. Thus it was found that saturated androgens were most inactive, while with one exception, the unsaturated derivatives promoted definite development. The presence of a double bond at Δ 4 or Δ 5 in the ring structure, and of an hydroxy group on the 3 or 17 position is apparently a requisite for activity.

EXPERIMENTAL.

Method I.

Preparation and Grouping of Experimental Animals.

Fifty male guinea-pigs, all four months of age, were castrated under light ether anaesthesia and, after an interval of fourteen days, weighed and allotted to five groups of ten animals each, so that the average weight of each group was approximately the same. A further ten males were maintained as controls.

The animals were housed in separate cages under identical conditions, and treated for a period of eight weeks as follows:

Group I. Castrate males fed a "normal" diet of 300 gm. fresh, green grass, plus 15 gm. supplementary ration[2] daily. Untreated.

Group II. Castrate males supplied with 30 gm. dehydrated potent subterranean clover, and in addition 15 gm. supplementary ration per day.

Group III. Castrate males fed a "normal" diet as for Group I, but receiving also intramuscular injections of 5 μg. stilboestrol in 0·1 ml. peanut oil every second day.

Group IV. Castrate males also maintained on a "normal" diet of grass and dietary supplement, but injected intramuscularly with 1 mg. testosterone propionate in 0·1 ml. peanut oil every alternate day.

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[2] See Diet.

Group V. Castrate males fed 30 mg. dehydrated subterranean clover plus 15 gm. supplement, and receiving also intramuscular injections of 1 mg. testosterone propionate in 0·1 ml. peanut oil every alternate day.

Group VI. Entire male animals fed the same ration as Groups 1, III, and IV untreated. In addition every animal was given 70 mg. ascorbic acid dissolved in the drinking water, at weekly intervals throughout the experimental period. All guinea-pigs had access to separate drinking bottles containing 120 ml. water, and these were renewed twice daily.

The Diet.

It was previously reported (East *et al.*, 1949) that fresh green clover proved fatal to guinea-pigs within two days, and that in an endeavour to minimize these fatalities the material was air-dried at 100°–130° F. and stored in four gallon air-tight tins. In addition a supplementary ration consisting of autoclaved bran 45 p.c., rolled oats 25 p.c., dried skim milk 30 p.c., moistened with a little water was provided. This enabled the animals to be kept alive indefinitely.

Materials Used.

The particular Dwalganup subterranean clover used in this experiment was Sample 3089 (1948 Series) from Paddock P. 77, grown at Beverley in moderately heavy soil over red and yellow mottled clay, and cut late in September just prior to wilting. The oestrogenic activity of the sample was measured by the mouse injection technique developed by Robinson (1949) and 100 gm. of the clover was found to have a potency equivalent to 10 μg. stilboestrol.

Daily intake figures for each guinea-pig calculated from the residue weights, showed that the clover was well consumed, the animals in Group II eating an average of 27·7 gm./pig/day, and those of Group V 27·9 gm./pig/day. The members of the second group therefore received a total of 155 μg., and of Group V, 156 μg. stilboestrol equivalent over the eight weeks period. As the animals comprising Group III were given 0·1 ml. injections of 5 μg. (0·005 mg.) stilboestrol in peanut oil three times every week, their total oestrogen intake was 120 μg. in fifty-six days. Hence the guinea-pigs in Groups II and V were receiving slightly more oestrogen than those of Group III. This is quite different from the conditions of the original experiment where, due to the low potency of the clover, the stilboestrol-injected animals were receiving rather more than twice the oestrogenic potency of the other two groups.

The androgen-oestrogen ratio in Group V was 154 μg. testosterone to 1 μg. stilboestrol.

Measurement.

The lengths of the right and left nipples of all guinea-pigs were determined at the commencement and end of the experiment, using vernier calipers measuring to 0·1 mm. The average initial and final lengths for each group were computed and the values analysed to indicate any significant differences due to treatment given.

Results.

From analysis of the data (Table 1) it appears that oestrogen treatment either in the form of clover ingested (Group II) or stilboestrol injected (Group III) caused a most definite increase in nipple length over the castrate condition (Group I), significant at the 1 p.c. level. Moreover there was no significant difference in the final mean values of these two groups, i.e. the clover and stilboestrol exerted a similar effect. There was no change in nipple length which could be attributed to castration.

Administration of testosterone propionate alone effected an apparent diminution of the castrate nipple but the decrease proved to be non-significant. When given conjointly with

clover to Group V the resultant teats were considerably smaller than those of the animals comprising Group II, but not as minute as in the entire or castrated guinea-pig. The protective action of the androgen was here manifested, although insufficient was supplied to give complete neutralization.

The above data were first analysed using covariance, but this method gave no greater precision. This indicated that either the original nipple lengths had little bearing on the final dimensions attained, or that they did not show sufficient variation to affect the ultimate result.

TABLE 1.

Effect of treatment on nipple length of the male guinea-pig.

Group	Treatment	Mean original length (R + L) mm.	Mean final length (R + L) mm.	Mean increase in length mm.
I	Castrated— Untreated	1·955	2·420	0·465
II	Castrated— Clover diet	1·815	5·210	3·395
III	Castrated— Normal diet + stilboestrol	2·200	5·500	3·300
IV	Castrated— Normal diet + testosterone	1·820	1·820	0·0
V	Castrated— Clover diet + testosterone	1·600	3·885	2·285
VI	Entire Untreated	2·205	3·340	0·135

Differences required for significance: 5 p.c., 0·56; 1 p.c., 0·74.

An attempt was also made in this experiment to demonstrate that the rate of teat growth relative to the rate of body growth was isometric in the mature animals of Group VI.

The obvious standard for body size with which to compare the teat length was of course, body length. Practical measurement of the latter value is unreliable, so the one third power of the body weight $\sqrt[3]{W}$ was adopted, since it is probable that the rate of increase in this function is approximately proportional to the rate of increase in body length (Bottomley and Folley, 1938). No linear relationship was obvious when the log. teat length (mm.) was plotted against log $\sqrt[3]{\text{body wt. (gm.)}}$. Examination showed that the animals employed were mature as judged by the original weights of the guinea-pigs used by Bottomley and Folley, and it was presumed that if, as stated by these investigators, the teat of the immature male guinea-pig grew isometrically with the body as whole, the attainment of maturity destroyed this relationship.

Method II.

This experiment was planned to indicate the periods of time necessary for minimal and maximal tissue response to a subterranean clover diet. In addition further data were obtained regarding the suitability of nipple lengths as criteria of treatment differences.

Preparation and Grouping of Experimental Animals.

Thirty male guinea-pigs were castrated and allotted to three groups of ten animals each as previously.

The Diet.

The animals comprising the three groups were housed separately and fed 30 gm. dehydrated clover and 15 gm. supplementary ration daily for periods of two, four, and six weeks respectively. In addition each pig received 70 mg. ascorbic acid per drinking water at weekly intervals.

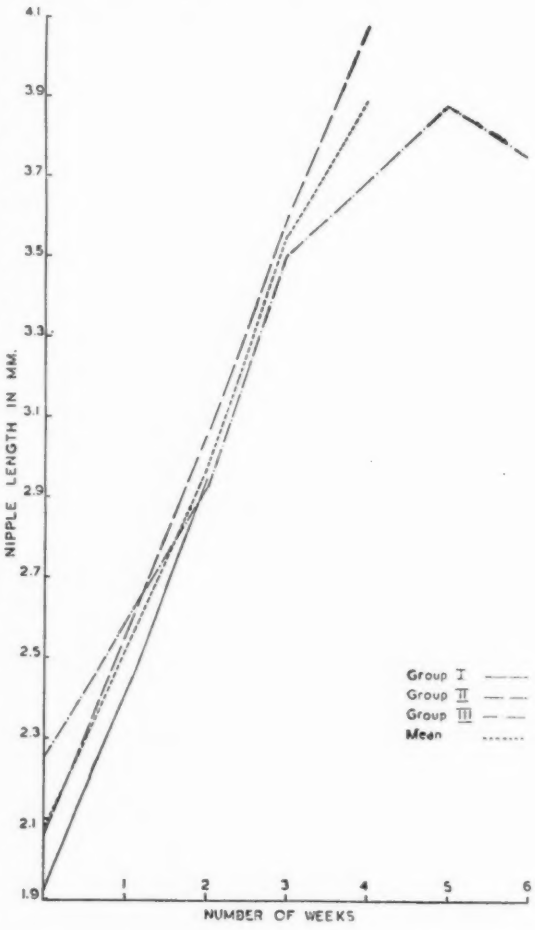
Materials Used.

The clover here employed was assayed for oestrogenic potency as before and 100 gm. of the material found to have a potency of 6·7 μg. stilboestrol.

Daily intake figures for each guinea-pig showed that the clover was consumed in daily amounts varying with the experimental period. Apparently the change from a lush green diet was severe and a certain time had to elapse before maximum intake was attained and held. Table 2 presents the average daily and total clover intake for the three groups and the calculated total stilboestrol equivalent received.

Measurement.

The lengths of the right and left nipples of all guinea-pigs were determined at the beginning of the experiment, at the end of two weeks (Group I), and thereafter at weekly intervals until slaughter.



Results.

Scrutinising the data presented in Tables 3 and 4, it was seen that the effect of the oestrogenically potent clover was obvious as early as fourteen days. The greatest increase occurred at one month, there being a difference between the mean final lengths of Group I and II significant at both the 5 p.c. and 1 p.c. levels. Groups II and III showed no difference in nipple lengths. After plotting the graph illustrated in Fig. 1 it was concluded that there was a substantial increment in teat length up to four weeks and that thereafter only a slight increase or even a diminution occurred.

TABLE 2.

Clover intake and clover equivalents.

Group	Exptl. period (days)	Av. clover intake gm./pig/day	Av. total clover intake gm./pig	Total equivs. stilboestrol consumed µg./pig
I	14	16·5	231·1	15·4 (15)
II	28	20·2	565·6	37·9 (38)
III	42	23·3	978·6	65·6 (66)

TABLE 3.

Effect of subterranean clover diet on nipple lengths of male guinea-pigs.

Group	Treatment	No. days	Mean original length mm.	Mean final length mm.	Mean increase in length mm.
I	30 gm. Clover/day	14	1·925	2·945	1·020
II	30 gm. Clover/day	28	2·055	4·075	2·020
III	30 gm. Clover/day	42	2·255	3·750	1·495

Differences required for significance: 5 p.c., 0·636; 1 p.c., 0·859.

TABLE 4.

Effect of subterranean clover diet on the mean nipple length of the male guinea-pig.

Group No.	29/9/48 Original length mm.	†12/10/48 Mean length mm.	19/10/48 Mean length mm.	26/10/48 Mean length mm.	2/11/48 Mean length mm.	9/11/48 Mean length mm.
I	1·925	2·945 *(1·020)				
II	2·055	3·050 (0·995)	3·600 (0·559)	4·075 (0·475)		
III	2·255	2·915 (0·660)	3·500 (0·585)	3·690 (0·190)	3·875 (0·185)	3·750 (—0·125)

* The figures in brackets represent the mean increases in nipple length during the experimental periods indicated.

† The first measurements were made 14 days after the start of the experiment and thereafter at weekly intervals.

DISCUSSION.

The results having shown that the nipple in the castrate male reacted to both known oestrogens and oestrogenic (clover) substance in an identical and measurable manner, consideration was given to its utilization as an assay procedure. The two techniques most generally employed to estimate the relative efficacy of oestrogenic preparations are the vaginal smear test and the uterus weight method. Using the first, a qualitative estimation of hormone reactivity is obtained, and this test is often employed in laboratory practice. The second method provides a quantitative measure of potency, and is so reliable that discrepancies due to diverse interpretations are few. Then in 1939 Fierz and co-workers added to these two techniques a third, which they termed the "Lokalen" Nipple Test (L.N.T.), involving as it does unction of the nipple of entire male guinea-pigs with test solutions.

These investigators found that it was possible to measure the nipple length in a simple manner, and that the increase of this organ after application of female sex hormones was thus easily determined. From such beginnings an assay method of some delicacy has been developed, Jadassohn, Fierz-David and Ruffoni (1940) showing that a linear relationship exists between the lengths of the guinea-pig nipple and the log. of the dose of oestrone, using solutions containing 0·05 μg.–0·2 μg. oestrone/ml. The test is sensitive also to artificial oestrogens such as diethylstilboestrol, to Anol and Anol derivatives, and human or animal urine. The L.N.T. according to the literature gives results concurrent with those obtained using either the vaginal smear or the uterine weight methods. Jongh and Laquer (1939) state that in practice the vaginal smear test works with a maximum error of 25 p.c., and in this respect the quantitative L.N.T. is equally efficacious. Even when adequate data are accumulated it is only known that, judging by the nature of the smear at a given number of hours after the first injection, compound A is more or less active than compound B. This in effect disregards possibilities of difference due to variations in the time course of their reactions, which potent substances may very well exhibit. Further, the conventional Allen-Doisy technique requires the subcutaneous injections of oestrogens, and the rate at which these compounds are absorbed, and their solubility in body fluids, become complicating factors. The chief objection to the uterine weight procedure is the necessity for killing the experimental animals.

What then are the possibilities of applying the L.N.T. as an assay procedure to determine the potency of subterranean clover? Immediately there arises the question of pituitary intervention in mammary growth, for it is essential that the assay technique be as specific and unequivocal as is practicable. Much controversy still exists on this point, and while local experimental evidence is naturally desirable, the burden of current research indicates that pituitary intervention may be discounted. The fact has been well attested that oestrogens applied directly to the mammary area in man, mice and monkeys and rabbits, can induce growth of the treated region without influencing other normally susceptible organs (Nelson, 1941; Gardner and Chamberlin, 1941). Further, if oestrogen (0·05 mg. oestrone/ml. 95 p.c. alcohol for 75 days) is applied percutaneously to one nipple of an immature rhesus monkey, a local and unilateral response is observed (Speert, 1940). Similar observations have been made by Fierz (1939) and Sür (1948) using the guinea-pig.

Hence it appears that the L.N.T. of Fierz is quite as practicable as the other two more common techniques. As the period required for appreciable increases in nipple length to appear, using ingested clover, is too long for con-

venience, and as the nipple is extremely sensitive to such stimuli, it is probable that unction of the nipple could be incorporated into an assay technique. Owing to the fact that castration does not affect the teat length entire animals could be employed, which eliminates pre-experimental preparation. Measurement of the nipple with vernier calipers is simple and does not necessitate slaughter, while local application greatly reduces the amount of active material required. Moreover, the teat is very sensitive to extremely dilute hormone solutions, and it is thus possible to apply materials which would not otherwise be adaptable to injection—e.g. alcoholic solutions.

There are certain disadvantages which must also be taken into account. Once the method of unction is utilized, the solutions applied must be in extract form. This promptly introduces the factor of loss, or at least change in potency due to extraction methods employed. However, the uterine weight procedure now being used (Robinson, 1949) is subject to the same criticism. The peculiar features of this oestrogenic effect consequent on subterranean clover feeding may, of course, be due to changes in the material during digestion or circulation in the animal body, and local application would not simulate such conditions. Nevertheless, it is suggested that all the evidence produced is distinctly favourable for the investigation of this L.N.T. technique.

SUMMARY.

An investigation has been made, in conjunction with other work, into the reaction of the nipple in the castrate male guinea-pig to a diet of oestrogenically potent subterranean clover.

Oestrogen treatment either in the form of clover ingested or stilboestrol injected caused a similar and statistically significant increase in nipple length over the castrate condition. The effect of the clover diet was obvious at fourteen days, the greatest increase occurring at one month.

There was no change in nipple length which could be attributed to castration, nor did administration of testosterone propionate have any effect on the castrate nipple.

When this androgen was given conjointly with a clover diet, however, the resultant teats were considerably smaller than those of the guinea-pigs receiving clover alone. The protective action of the testosterone was here manifest although insufficient was supplied to give complete neutralization.

A discussion is given as to the possibility of developing such a nipple length test as an assay procedure, by future trials designed specifically for this purpose.

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THE INHIBITION OF THE OXIDATION OF ALANINE BY ACRIDINES AND RELATED COMPOUNDS

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Investigations on the effect of acridines on enzymatic activity have, in general, been confined to the action of particular acridines on various enzyme systems or the effect of different acridines on general respiration, Wright and Sabine (1944), Haas (1944), Ferguson and Thorne (1946), Hellerman, Lindsay, Bovarnick (1946), and Albert and Marshall (1948). In this series of experiments the effects were compared of acridines and related compounds, of widely varying antibacterial activity, on the oxidation of alanine by resting cell suspensions of *Bact. coli.*

EXPERIMENTAL.

The strain of *Bact. coli* used in these experiments was that used by Albert, Rubbo *et al.* (1942), (1945), in their investigations on the acridine series. The resting cell suspensions were prepared by harvesting in Ringer's solution the growth from 24 hour cultures of *Bact. coli* grown on papain digest agar in Roux bottles at 37° C. The suspensions were washed twice in Ringer's solution and the opacity adjusted to give approximately 1 mg./ml. dry weight using the Evelyn photoelectric colorimeter.

Drug solutions were made in M/40 phosphate buffer pH 7·8. The final concentration of drug in the flask was usually $1{\cdot}1 \times 10^{-4}$ Molar. A few experiments were performed using $2{\cdot}2 \times 10^{-4}$ M drug.

The experiments were carried out using the Warburg microrespirometer. The contents of the flasks for the investigation of a drug are set out in Table 1.

TABLE 1.

Contents of Warburg flasks.

	Vol. (ml.)	Flasks			
		1	2	3	4
Suspension	1·0	+	+	+	+
M/10 PO_4 buffer pH 7·8	0·4	+	+	+	+
M/10 dl-alanine	0·3	—	+	—	+
Drug	0·3	—	—	+	+
20 p.c. KOH (in the cup)	0·2	+	+	+	+
Distilled H_2O to	2·5	+	+	+	+

The dl-alanine and drug were placed in the side arms of the flask and tipped in simultaneously after equilibration for 20 minutes. For each experiment the oxidation of the alanine was measured by subtracting the value obtained for the "endogenous" respiration (Flask 1) from that of the oxygen uptake in the presence of alanine (Flask 2). In accord with the work of Albert and Falk (1949) it was found that the oxygen uptake of the resting cell suspension was usually stimulated, to a greater or lesser degree, by the presence of the drug. Therefore in testing the effect of a drug, the "endogenous" oxygen uptake was measured in the presence of the drug (Flask 3). These results were subtracted from the results of the measurement of oxygen uptake in the flask containing cells, alanine and drug (Flask 4) to give the results plotted for the oxygen uptake curves.

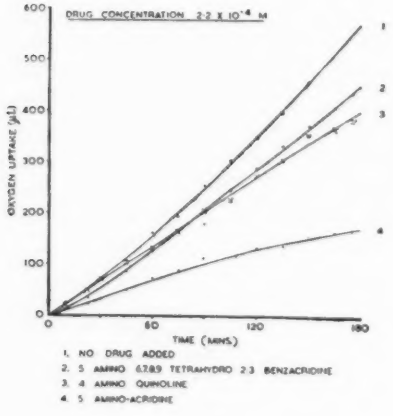
DRUG CONCENTRATION 2·2 X 10⁻⁴ M
OXYGEN UPTAKE (µl)
TIME (MINS.)
1. NO DRUG ADDED
2. 5 AMINO 6.7.8.9 TETRAHYDRO 2.3 BENZACRIDINE
3. 4 AMINO QUINOLINE
4. 5 AMINO-ACRIDINE

Fig. 1.

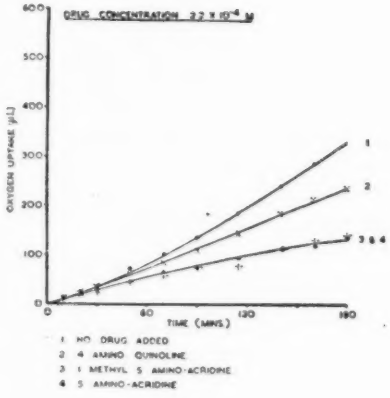

Fig. 2.

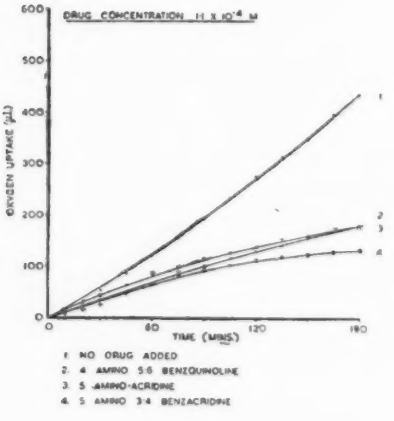
DRUG CONCENTRATION 1·1 X 10⁻⁴ M
OXYGEN UPTAKE (µl)
TIME (MINS.)
1. NO DRUG ADDED
2. 4 AMINO 5·6 BENZQUINOLINE
3. 5 AMINO-ACRIDINE
4. 5 AMINO 3·4 BENZACRIDINE

Fig. 3.

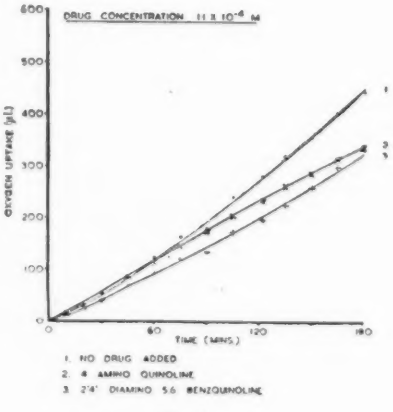

Fig. 4.

When it was attempted to compare a series of compounds it was found that the effectiveness of a given compound may vary considerably when tested on consecutive days using the same bacterial suspension. This is illustrated by a comparison of the results of two successive experiments using 5-amino-acridine, depicted in Figs. 3 and 5, in which the same bacterial suspension was used, one experiment being carried out 24 hours after the other. Therefore, it was not possible to compare quantitatively the results of compounds tested in different experiments. However, 5-amino acridine was included in each series of experiments to provide a standard of reference and, as far as practicable, compounds of widely differing antibacterial activity were tested in each experiment.

The compounds used were selected from the results of the extensive survey of Albert, Rubbo and Burvill (1949). A number of the more interesting compounds were insoluble at the pH and concentration used, or not available in sufficient quantity for testing.

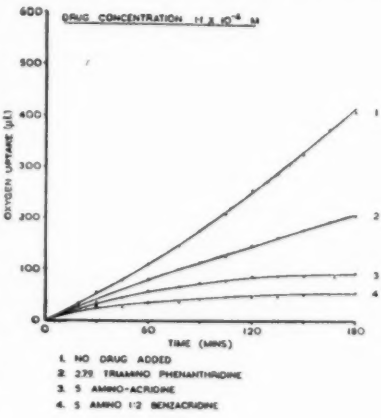

Fig. 5.

In Figs. 1, 2, 3, 4 and 5 are depicted the results from typical experiments. It will be seen that those drugs which have high bacteriostatic activity (see Table 2), inhibit the oxidation of alanine to a much greater degree than those which have low bacteriostatic activity. For example, Fig. 1 shows that 5-aminoacridine, which is bacteriostatic at a concentration of 1 in 40,000 inhibits the oxygen uptake markedly while 5-amino-6:7:8:9- tetrahydro-2:3-benzacridine and 4-aminoquinoline, which are bacteriostatic at concentrations of 1 in 10,000, and less than 1 in 5,000, respectively, inhibit to a much less degree. In Fig. 2 it is seen that 5-aminoacridine and 1-methyl-5-aminoacridine which have identical bacteriostatic activities against *Bact. coli* inhibit alanine oxidation to the same extent, while 4-aminoquinoline has lower activity.

Table 2 shows the results obtained for 12 compounds, together with their bacteriostatic activity against *Bact. coli*, and percentage ionisation at pH 7·8.

It is of interest that in the presence of 10^{-4} M 5-aminoacridine at 37° C. and pH 7·8, the suspension still contained viable organisms after 20 hours.

DISCUSSION.

The relationship between chemical structure, degree of ionization and bacteriostatic activity among certain heterocyclic bases has been fully investigated recently by Albert, Rubbo and Burvill (1949). It was shown that for maximal bacteriostatic activity, the drug molecule must be highly ionized at the pH at which the test is performed and also must have a certain minimal flat area, presumably to facilitate adsorption to the surface at the site of action. Similar factors apparently play an important rôle in the inhibition of alanine oxidation by *Bact. coli* with these compounds.

TABLE 2.

Substance	Reciprocal of highest diln. completely preventing visible growth of *Bact. coli* in 48 hours 37° C. in 10 p.c. serum broth (Albert, Rubbo *et al.* 1945), (Albert, Rubbo and Burvill, 1949)	Degree of inhibition of alanine oxidation	P.c. substance ionized at pH 7·8 20° C. (Albert, Rubbo *et al.* (1945); (Albert, Rubbo and Burvill 1949)
5-aminoacridine	40,000	Marked	99
1-methyl-5-aminoacridine	40,000	Marked	99
5-amino-3:4-benzacridine	20,000	Marked	94
5-amino-1:2-benzacridine	20,000	Marked	91
4-amino-5:6-benzquinoline	20,000	Marked	94
5-amino-6:7:8:9-tetrahydro-2:3-benzacridine	10,000	Slight	100
2:7:9-triamino-phenanthridine	5,000	Slight	94
5-amino-6:7:8:9-tetrahydro-acridine	<5,000	Slight	99
4-hydroxy-5-aminoacridine	<5,000	Slight	5
acridine	<5,000	Slight	0·1
2′:4′-diamino-5:6-benzquinoline	<5,000	Slight	0·1
4-aminoquinoline	<5,000	Slight	94
		Slight—>20 p.c. less inhibition than 5 amino-acridine	

The correlation between bacteriostatic activity and inhibition of oxidation of alanine among the acridines and related compounds may indicate that the bacteriostatic action of these compounds is related in some way to interference with the functioning of the enzyme system responsible for, or indirectly linked with, the oxidation of alanine. This is not incompatible with the hypothesis advanced by several workers, Haas (1944), Wright and Sabine (1944), Hellerman *et al.* (1946) and Sevag (1949), namely, that the mode of action of acridines may be concerned with interference with flavoprotein catalysis. From a consideration of the shape of the oxygen uptake curves it would seem that the more active compounds cause a progressively greater inhibition of enzymatic activity, possibly due to irreversible inactivation of enzyme by the drug. However, the curves obtained from the less active compounds tend towards straight lines, which may indicate that the inhibition produced by these compounds may be due to a reversible inhibition of the enzyme.

SUMMARY.

Investigation of the action of certain heterocyclic bases on *Bact. coli* shows a correlation between bacteriostatic activity and inhibition of alanine oxidation.

Acknowledgments. I wish to thank Professor S. D. Rubbo for his interest in this work and Miss L. Nutting for preparing the Figures. The compounds tested were those used by Albert, Rubbo *et al.* (1942), (1945), (1949), synthesized by Professor A. Albert and his colleagues.

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THE LIBERATION OF HISTAMINE AND HEPARIN BY TUBOCURARINE AND ITS CIRCULATORY EFFECTS IN THE DOG

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Considerable information about the release of histamine from tissues has been obtained from a study of the action of various toxic agents on the isolated perfused lungs of the guinea-pig (Kellaway, 1947). It is known that curare (Alam *et al.*, 1939) and tubocurarine (Schild and Gregory, 1947) liberate histamine from the skeletal muscle of the dog. Alam *et al.* (1939) found no histamine to be liberated from lungs of the dog in heart-lung preparations and they considered that the action of curare is confined mainly to skeletal muscle. On the other hand in circulatory shock in the dog caused by agents such as peptone or anaphylactic antigen which release histamine, constriction of hepatic venules by the histamine liberated from the liver is of outstanding importance.

This paper reports a study of the pattern of release of histamine from the guinea-pig's lungs by tubocurarine. It also describes the action of tubocurarine in the liver of the dog and the part which this plays in its circulatory effects.

METHODS.

Tubocurarine chloride (Burroughs Wellcome & Co.) was used. Extracts of tissues were made as described by Feldberg and Kellaway (1937a, 1937b) and histamine in them and in samples of perfusate was estimated on the isolated jejunum of the guinea-pig in an organ bath 8 ml. in volume and sometimes on the blood pressure of an atropinized cat under chloralose anaesthesia. Histamine is expressed in terms of the acid phosphate.

The lungs of the guinea-pig in an incubator at 37° C. were perfused as described by Feldberg and Kellaway (1937a). Tubocurarine was injected into the pulmonary cannula in volumes of 0·1 to 1·5 ml.

The dogs (usually after a subcutaneous injection of 10 mg./kg. of morphine) were anaesthetized with ether and then given 80 mg./kg. of chloralose intravenously. In most cases the vagi were divided, a cannula tied into the trachea and artificial ventilation established. The systemic blood pressure was recorded from a carotid artery. A plastic catheter passed from the jugular vein into the right ventricle was used for recording the right ventricular pressure. To record systemic venous pressure a similar catheter was passed into the jugular vein or into the right auricle and connected through a manometer filled with 25 p.c. sodium thiosulphate to a Brodie's tambour. The pressure in the portal vein was recorded in a similar

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manner, the catheter being inserted into the splenic vein; the spleen was removed. From other animals anaesthetized as above a portion of the liver was perfused through the portal vein as described by Feldberg and Kellaway (1937b). In some of these experiments the pressure in the portal cannula was recorded through a T-piece, manometer and tambour. Heparin titrations were carried out as described by Jaques and Waters (1941).

RESULTS.

Experiments on Guinea-pigs.

The Action of Tubocurarine on the Guinea-pig Jejunum.

Since the first or second sample of perfusate after the injection of tubocurarine was likely to contain the drug in a relatively high concentration, it was necessary to examine the effect of tubocurarine on the isolated gut of the guinea-pig. Small doses (1 to 100 μg.) when added to the organ bath were usually without action and on washing out some 10 to 30 sec. later, there was no change in the sensitivity of the gut to histamine. Larger doses (0·5 to 10 mg.) caused relaxation especially when the tone of the gut was high. Sometimes the relaxation was preceded by a very small contraction. On washing out the bath the response to histamine was depressed, the duration and degree of depression varying with the dose of tubocurarine and the time of contact with the preparation. With short periods of contact (5–10 seconds) the subsequent response to histamine was often potentiated.

When histamine (0·1 μg.) was added to the bath in a solution of Tyrode or inactive perfusate containing also 0·5 to 5 mg. of tubocurarine the expected response to histamine varied; sometimes it was scarcely affected, sometimes it was slightly potentiated (cf. Silva and Schild, 1949) and sometimes, especially with the larger doses, it was reduced to one-third or one-quarter. In the latter case boiling the solution restored the activity to about its expected value. Practically, however, the presence of tubocurarine did not present a difficult problem because the samples of perfusate likely to contain much of it were also the samples richest in histamine. An easily titratable amount of histamine was usually present in a volume which contained too little tubocurarine seriously to interfere. For example, in the experiment represented in Fig. 2 the first sample after injection could not contain more than 150 μg. of tubocurarine for every 0·1 μg. of histamine. In any case, the samples were boiled for 2 minutes, left in contact for only 5–10 seconds and matched against boiled solutions containing both histamine and an estimated amount of tubocurarine. For example, an experiment in which 10 mg. of tubocurarine were injected yielded 5 ml. of perfusate in the first four minutes. Clearly this sample could not contain more than 1 mg. of tubocurarine in each 0·5 ml. and when boiled it appeared to contain 0·1 μg. of histamine per 0·5 ml. when matched against a histamine solution. When matched against a boiled solution containing 1 mg. of tubocurarine for every 0·1 μg. of histamine the same result obtained.

Perfusion Experiments.

Perfusion of the right lobe was carried out at a rate of 0·5 to 2·0 ml. per min. and samples of perfusate were collected every two to four minutes for the first 20 minutes and every ten to fifteen minutes thereafter. Tubocurarine in doses ranging from 1·0 to 30 mg. released histamine, the output of which was maximal in the first four minutes and declined rapidly at first and then more slowly until it became undetectable in 10 minutes to an hour or more. In fourteen experiments the output in half an hour ranged from 0·36 to 8·9 μg. Fig. 1 illustrates the characteristic pattern of output in three experiments. Fig. 2 illustrates the result of another experiment in which the output is greater than in those shown in Fig. 1. It also

illustrates the usual finding that a second injection causes a much smaller output than the first. Usually the amount of histamine lost was not sufficient to enable one to detect a difference between the histamine contents of the injected right and the uninjected left lobes. There was little or no evidence of broncho-constriction following the injection of tubocurarine. Usually pulmonary ventilation was improved rather than impaired.

No significant amounts of muscle stimulating substances other than histamine were detected.

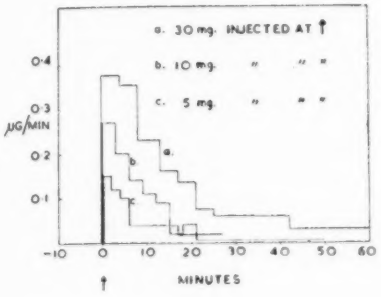

Fig. 1.

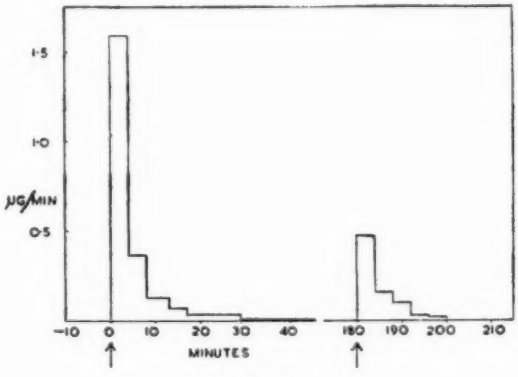

Fig. 2.

Fig. 1. Output of histamine in μg./min. from the isolated perfused lung of the guinea-pig in three experiments—(a), (b) and (c). The lungs contained respectively 70 μg., 28 μg. and 36 μg. per gm. of histamine.

Fig. 2. Output of histamine from isolated perfused lung of guinea-pig resulting from two injections (at arrows) of 10 mg. of tubocurarine. The lung contained 42 μg./gm. of histamine.

Experiments on Dogs.

The Effect of Tubocurarine on the Pressure in the Portal Vein.

Injections into the Portal Circulation.

When tubocurarine chloride in doses of 0·25 to 15 mg. is injected into a mesenteric vein, there is a fall in systemic arterial and venous pressures and a rise in pressure in the portal vein. The rise of portal and the fall of arterial blood pressures take place simultaneously about 20 seconds after the injection. The fall of systemic venous pressure takes place usually at the same time but occasionally did not begin to fall for a further 20 to 30 seconds. Fig. 3 shows the effect of injecting 4 mg. of tubocurarine into the mesenteric vein of a dog weighing 12·6 kg.; systemic arterial and venous pressures fell simultaneously with a sharp rise of pressure in the portal vein. These circulatory changes are associated with swelling and congestion of the liver and commonly with vigorous gastric peristalsis and accumulation of acid gastric juice. The secondary rise of portal venous pressure shown in Fig. 3 occurred quite commonly and can be seen also in Figs. 4 and 5. A second injection of 4 mg. some 30 minutes after the first caused a much smaller response in all three tracings. In some experiments the repetition

of a dose of this order caused only a trivial change in portal pressure. This is illustrated in Fig. 4 which shows the effect of two successive injections of 2 mg. of tubocurarine in the mesenteric vein of a dog weighing 12 kg. In such experiments a third injection of a much larger dose elicited no response but histamine still evoked a rise of portal pressure. Amounts such as 0·25 mg. which gave slight changes only, could be repeated two or three times without significant tachyphylaxis.

When, following injection into a mesenteric vein, the fall of arterial blood pressure was very great and prolonged, the pressure in the portal vein after its initial rise settled to a lower level than it was before the injection, as illustrated by Fig. 5.

Injections into a Systemic Vein.

Following systemic intravenous injection of 2 mg. or more a fall of arterial and venous pressures also takes place but the effect on the portal venous pressure is variable. Most commonly it begins to fall at the same time as the arterial blood pressure and the fall may be interrupted by a rise which is usually small and transient but sometimes larger and prolonged (cf. Figs. 6 and 7). Sometimes the pressure in the portal vein rises without initially falling. In any case when a rise of portal pressure follows intravenous injection it regularly begins some 20 to 30 seconds after the arterial pressure has begun to fall. Because of the change in sensitivity a strict comparison of the effects of intraportal and intrasystemic venous injections, could not be made in the one animal. Nevertheless doses which caused trivial effects on the pressure in the portal vein when given intravenously, caused a rise of portal venous pressure on subsequent intraportal injection and even when the systemic intravenous injection caused a rise of portal pressure subsequent intraportal injection commonly caused an even greater rise. Fig. 6 shows the effect on the arterial and portal venous pressures of 2 mg. of tubocurarine given intravenously and intraportally into a dog weighing 8 kg., the letters I.V. and I.P. denoting the respective routes. The fact that the rise of portal pressure is greater on injection into the portal circulation than it is on injection into the systemic circulation indicates that the rise of pressure in the portal vein is due mainly to an action of the drug in the liver itself.

Effect of Benadryl.

On analogy with the effects of peptone or anaphylaxis, these results are strongly suggestive of the liberation of histamine from the liver. This is supported by experiments with anti-histamine drugs. Fig. 8 shows the effect of 0·25 mg. of tubocurarine given both intravenously and intraportally into a dog weighing 15 kg. It can be seen that the systemic intravenous injection of tubocurarine caused no significant change in either the pressure in the portal vein or in the arterial pressure, whereas 50 μg. of histamine given intravenously caused a fall of arterial pressure and a small rise of portal pressure. Portal intravenous injection of these drugs showed that tubocurarine (0·25 mg.) now caused a rise of portal pressure greater than that caused by 50 μg. of histamine acid phosphate. Between the third and fourth panels 50 mg. of benadryl were given intravenously. It can be seen that the responses to the intraportal injection of both tubocurarine and histamine are greatly reduced. However, 4 mg. of tubocurarine still caused a considerable rise in portal pressure. This experiment illustrates the general finding that small doses of tubocurarine are antagonized by benadryl to the same degree as is histamine itself but that larger doses still cause a rise in portal pressure even in the presence of benadryl. Very often large doses of tubocurarine caused such great and prolonged changes in the circulation (e.g. Figs. 3 and 5) that no match with histamine was possible and in any case because of the change in sensitivity no comparison of their effects could be made before and after benadryl. Such doses are probably equivalent to the injection of one or several milligrammes of histamine. It is difficult to abolish the effect of such large doses of histamine by means of an anti-histamine drug. (Dews and Graham, 1946; Marsh and Davis, 1947; MacIntosh and Paton, 1949.)

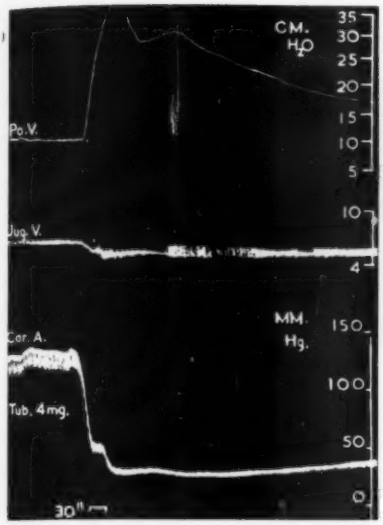

Fig. 3.

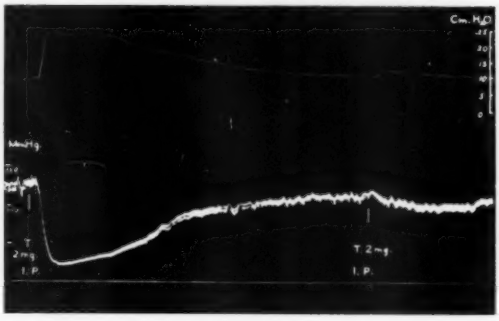

Fig. 4.

Fig. 3. Records of pressures in portal vein (upper tracing), jugular vein (middle tracing), and carotid artery (lower tracing) in a dog weighing 12·6 kg. Time in half minutes. Details in text.

Fig. 4. Record of pressures in portal vein (upper tracing) and carotid artery (lower tracing) of a 12 kg. dog. Injections into a mesenteric vein. Details in text.

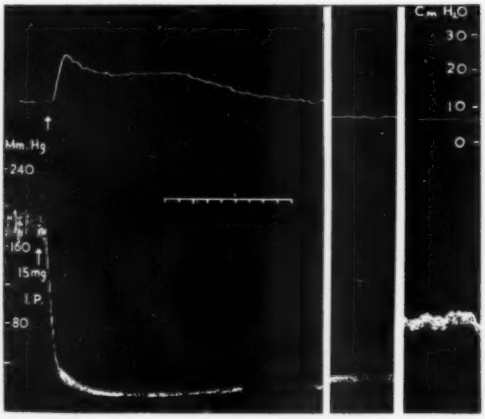

Fig. 5.

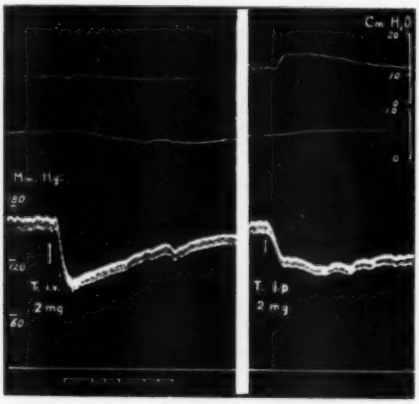

Fig. 6.

Fig. 5. Dog 10 kg. (Experiment number 5 of Table 1). Injection into a mesenteric vein of 15 mg. of tubocurarine. Interval between panels: 5 and 15 minutes respectively.

Fig. 6. Dog 8 kg. Records as in Fig. 3. Injections into femoral vein or into mesenteric vein as indicated by letters I.V. or I.P. respectively. Time in half minutes. Details in text.

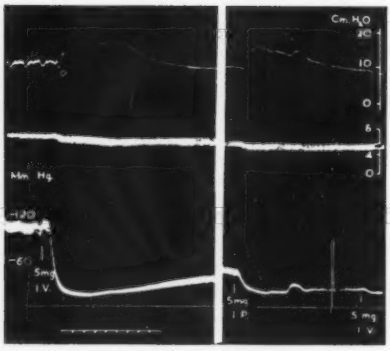

Fig. 7.

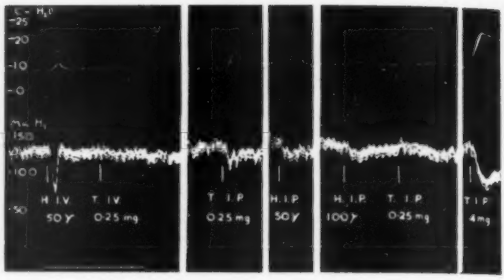

Fig. 8.

Fig. 7. Records as in Fig. 3 of dog weighing 10 kg. Tubocurarine was injected at the strokes, the letters I.V. or I.P. denoting the routes of administration as in Fig. 6. Time in half minutes.

Fig. 8. Dog 15 kg. Records as in Fig. 4 and lettering as in Fig. 6. Histamine is denoted by H and tubocurarine by T. Time in half minutes. Details in text.

The Release of Histamine from the Liver.

More direct evidence that tubocurarine releases histamine from the liver is provided by a comparison of the histamine content of two pieces of liver removed (a) before and (b) after the intraportal injection of tubocurarine.

In Vivo *Experiments.*

The experiment was done in the usual way and the pressures in the portal vein and carotid artery recorded. A piece of liver weighing about 2 or 3 gm. was then removed, an intraportal injection of tubocurarine made and at an interval varying from 15 minutes to 80 minutes a second piece was obtained. Before extraction the liver was cut up into small pieces and the blood allowed to drain out as far as possible. This was done because of the possible congestion of the second sample, but in any case this had usually subsided by the time the piece was removed. The results are set out in Table 1.

TABLE 1.

In vivo *experiments.*

No.	Dose of tubocurarine	Mass of liver	Histamine content (μg./gm.)		Total loss
			Before	After	
1	3 mg.	300 gm.	55	36	5·7 mg.
2	4 mg.	325 gm.	50	39	3·6 mg.
3	5 mg.	340 gm.	31	32	—
4	10 mg.	305 gm.	13	7·5	1·7 mg.
5	15 mg.	340 gm.	70	56	4·7 mg.
6	14 mg.	300 gm.	112	62	15·0 mg.

In all except one experiment the first sample has the higher content and in all the others the loss is equal to or greater than 20 p.c. The natural difference in the histamine content of two pieces of liver (Ojers *et al.*, 1941) could not account for this difference.

In Vitro *Experiments.*

Injection of tubocurarine into the fluid perfusing a piece of liver at rates varying from 1·5 to 6 ml./min. never caused a loss of more than a few microgrammes of histamine. This was not due to destruction of histamine in the perfusate which did not contain histaminase in significant quantities, and in any case the histamine content of the liver showed no detectable change after perfusion. The results of five experiments are set out in Table 2.

TABLE 2.

In vitro *experiments.*

No.	Perfusate	Dose of Tubocurarine	Mass of liver	Histamine content	Loss in perfusate
1	Tyrode	1 mg.	110 gm.	70 μg./gm.	<0·9 μg.
2	Tyrode	10 mg.	120 gm.	28 μg./gm.	5·4 μg.
3	Tyrode	10 mg.	145 gm.	30 μg./gm.	Nil
4	Tyrode	5 mg.	160 gm.	135 μg./gm.	3·7 μg.
5	Heparinised blood	5 mg.	79 gm.	60 μg./gm.	Nil

In three of these experiments the pressure in the portal cannula was recorded. The injection of tubocurarine caused only trivial changes in the rate of outflow and in the portal pressure. For example, in Experiment 3 the injection of 10 mg. of tubocurarine caused no change in pressure. The subsequent injection of 100 μg. of histamine caused a rise of up to 3 cm. of water, lasting for 15 minutes.

The Release of Heparin from the Liver.

The discrepancy between the results of the *in vivo* and the *in vitro* experiments recalls the similar discrepancy which also exists with injury with peptone, anaphylactic antigen and ascaris extracts. These agents release large quantities of histamine from the liver of the intact dog but only small amounts when the liver is perfused with Tyrode solution or heparinized or defibrinated blood (Kellaway, 1947, Rocha e Silva *et al.*, 1946a, 1946b, 1947.) This similarity raised the possibility that like these agents, tubocurarine liberates heparin from the liver. In the early experiments changes in coagulation were not investigated because a small amount of heparin had been introduced into the recording cannulae. In five later experiments (that of Fig. 3 and numbers 3 to 6 of Table 1) where this was not done, clear evidence of change in coagulability was observed. In the experiment of Fig. 3, and numbers 4 and 5 of Table 1, blood withdrawn from a carotid artery before injection clotted in 3–10 minutes; blood withdrawn 10–15 minutes after injection was unclotted after 3 hours at room temperature and still unclotted after a further 24 hours in the refrigerator. In experiment 3 of Table 1 in which no loss of histamine was detectable the clotting time increased to 1 hour. In experiment number 6 blood withdrawn 5, 15 and 80 minutes afterwards was left at room temperature; the first two samples were still liquid after 5 hours and clotted within the next 12 hours in the refrigerator; the last sample clotted in 2 hours.

The failure of coagulation is not due to the absence of fibrinogen because the blood clotted promptly on the addition of thrombin (Parke Davis and Co.). That the anticoagulant is heparin is indicated by the fact that clotting took place on the addition of an appropriate amount of protamine sulphate. A typical titration carried out as described by Jaques and Waters (1941) gave the following result. In experiment 4 of Table 1, blood was removed 4, 10 and 22 minutes after injection. All samples were liquid after 3 hours at room temperature. The amounts of protamine required to reduce clotting time to its lowest value were respectively 0·02, 0·015 and 0·02 mg./0·5 ml. of blood.

No changes in coagulation occurred in two experiments in which tubocurarine (2 and 20 mg. respectively) was injected into a systemic vein. The first experiment is that of Fig. 6. The subsequent intraportal injection of 2 mg. also had no effect on coagulation and a further 8 mg. given intra-portally had no effect either on coagulation or on the pressure in the portal vein. No loss of histamine could be detected from the liver; in the second experiment the intravenous injection of 20 mg. of tubocurarine caused a rise of portal pressure and a profound fall of arterial blood pressure. The rise of portal pressure (but not the depressor effect) was equivalent to that caused by the intra-portal injection of 200 μg. of histamine. No coagulative change occurred; the histamine content of the blood had increased from less than 0·04 μg. per ml. to 0·1 μg. per ml. There was no detectable loss from either the gastrocnemius which contained 3 μg./gm. nor from the liver which contained 150 μg./gm. Sixty minutes later 20 mg. of tubocurarine were injected into a mesenteric vein. This was without effect either on the portal pressure, the histamine content of the liver or on the coagulation time.

The Part Played by the Liver in the Circulatory Effects of Tubocurarine.

The liver does not play the dominant rôle in the circulatory action of curare because injections intravenously which cause a big depressor effect are often unassociated with significant change in the pressure in the portal vein (e.g. Fig. 6). A fall of blood pressure was also observed in the eviscerated animal. Further information about the circulatory effects are provided by the following experiments.

The Effect of Tubocurarine on the Blood Vessels of the Hind Limb.

Both the femoral and sciatic nerves were divided, tubocurarine injected into the femoral artery through the cut stump of a muscular branch and the outflow from the femoral vein measured by the method of Feldberg and Schilf (1930). In one experiment the outflow was 10·5 ml./0·5 minutes before the injection of 0·5 mg. of tubocurarine. One minute afterwards it had reached the peak value of 20 ml./0·5 minutes, following which it declined until 8 minutes later it was 11 ml./0·5 minutes. Since ganglia are absent from the region of distribution of the drug, and in any case the nerves to the muscles had been cut, the vasodilatation is not due to an effect on ganglia. In view of the observations of Schild and Gregory (1947) and Alam *et al.* (1939) it is attributable to the release of histamine.

The Part Played by Ganglionic Block.

The results already described indicate that other factors than ganglionic blockade are responsible for the fall in blood pressure. This of course does not exclude the possibility that ganglionic paralysis plays a part. Indeed it would be surprising if it were not so for curare in large doses does block ganglionic transmission (Langley and Dickenson, 1890; McIntyre, 1947).

To examine the part played by ganglion paralysis, the contraction of the nictitating membrane and the arterial blood pressure was recorded in dogs. A vagus nerve was divided near the superior cervical ganglion leaving the sympathetic fibres intact. In one typical experiment

the preganglionic fibres in the vagosympathetic were stimulated at 10 per second with square waves 1 m.sec. in duration. On the administration of 2 mg. or 8 mg. of tetra ethyl ammonium iodide, the nictitating membrane relaxed as rapidly as if stimulation had ceased, whereas tubocurarine caused a smaller, less rapid and more prolonged relaxation. For a given fall of blood pressure the effect of tubocurarine on the nictitating membrane was much less than that of the ganglionic blocking agent—tetra ethyl ammonium iodide, indicating that the depressor action of tubocurarine is not mainly due to ganglionic block but showing that it possibly contributes. It is probable, however, as was shown by Langley and Dickenson (1890) that the vasomotor cells are more readily paralysed by curare than are the ones which supply the nictitating membrane. Nevertheless, in this experiment, both 2 mg. and 8 mg. of tetra ethyl ammonium iodide are probably exerting a maximal effect on the vasomotor cells, for in each case the fall in blood pressure is about the same. The effect of 8 mg. is merely longer in duration. On the other hand 10 mg. of tubocurarine caused a considerably greater fall in blood pressure than 2 or 8 mg. of tetra ethyl ammonium iodide.

The Pressure in the Right Ventricle.

On the intravenous injection of tubocurarine the pressure in the right ventricle falls together with the fall in systemic arterial pressure.

DISCUSSION.

The pattern of output of histamine from the perfused lungs of the guinea-pig is distinctly different from that caused by snake venoms or bacterial toxins (Feldberg and Kellaway, 1937a, 1938a, 1938b). It resembles more that seen in anaphylactic or peptone injury.

This pattern of output of histamine can be interpreted as a diffusion process as Rocha e Silva and Schild (1949) have recently done for the loss of histamine from the rat's diaphragm immersed in a solution of tubocurarine. In the latter case the concentration of tubocurarine may be made so great that for practical purposes sufficient penetrates the cell to exert its maximum effect within a few minutes and thereafter the histamine which has been unbound is lost at a rate determined by its rate of diffusion through the mass of tissue. In the perfusion experiment, on the other hand, no attempt has been made to control the rate of diffusion into the cell of tubocurarine which in any case is only present in the perfusing fluid momentarily. But for any experiment the injection of tubocurarine causes a small proportion of the total histamine to be set free and this continues to enter the perfusate for periods varying from 10 minutes to an hour or more. The diagram relating the rate of appearance of histamine to time is consistent with the view that it is set free within a few minutes of injection and the subsequent time-course is a function of the rate at which it can pass from the cell into a perfusion fluid which is virtually histamine free. If time is plotted against the logarithm of the rate of loss of histamine the points lie roughly along a straight line.

So far as the circulatory effects of tubocurarine are concerned, as McIntyre (1947) states, "the utmost confusion prevails." Its depressor action has been ascribed, not always with justification, to a cardiotoxic effect (McIntyre, 1947),

to the release of histamine (Everett, 1948), to a loss of muscle tone (Everett, 1948), and to ganglionic block.

These experiments show clearly that the depressor effect in the dog is associated with a fall of venous pressure and cannot therefore be cardiac in origin. Also it is not due to obstruction in the pulmonary circulation. Injections into the portal vein release histamine from the liver, causing the pressure in the portal vein to rise, and when the dose of tubocurarine so given is small this effect may determine a fall of blood pressure which would not take place on systemic intravenous injection. With larger amounts of tubocurarine injected into systemic veins, a fall of arterial pressure takes place but this is not due to obstruction in the liver because the portal pressure may fall or show trivial pressure changes. Probably some hepatic obstruction does occur as is indicated by experiments in which a rise of portal pressure followed intravenous injection; at any time the pressure in the portal vein will be the resultant of factors causing it to rise, such as hepatic obstruction, and of factors causing it to fall, such as a decreased entry of blood from the arteries.

Since neither hepatic nor pulmonary vascular obstruction nor a direct cardiotoxic action is responsible for the depressor effect it must be attributable to peripheral changes. The drug causes a decreased resistance in the hind limb which is not due to ganglion block and not due to loss of muscle tone which had been already abolished by nerve section. In view of the similar observations of Alam *et al.* (1939) this is probably due to the release of histamine. The experiments with the nictitating membrane indicate that ganglionic block does play a part when large doses are used, but it is much less important than is the case with a ganglion blocking agent such as tetra ethyl ammonium iodide.

Since the portal pressure still responds to histamine after it has become insensitive to tubocurarine, the loss of sensitivity cannot be due, as Alam *et al.* (1939) suggest, to the antihistamine action of liberated histamine. The experiments with the lungs indicate that there is decreased ability to liberate histamine. Whatever may be the mechanism of this tachyphylaxis is obscure. Only a fraction of the total extractable histamine has been liberated when the preparation becomes refractory and it is well known (Kellaway, 1947) that other agents such as cobra venom and bacterial toxins are capable of liberating much more. The mode of action of such agents must be different from that of tubocurarine.

There are certain effects of tubocurarine which resemble those due to peptone or anaphylaxis. In addition to the pattern of liberation of histamine from the lungs of the guinea-pig and the release of histamine from the dog's liver, there is the loss of sensitivity to repeated injections in the dog, the discrepancy between *in vivo* and *in vitro* experiments with the dog's liver and the observation which it led to, of the release of heparin. MacIntosh and Paton (1949) who showed that histamine and heparin were liberated from the dog's liver by certain organic bases, were also impressed with their pharmacological similarity

to peptone and anaphylactic antigen. There are, however, certain differences, so far as tubocurarine is concerned, such as the absence of contraction of the guinea-pig jejunum and the weak or absent broncho-constriction in the perfused lungs but perhaps this may be due to the antagonism of curare to the action of histamine on the smooth muscle. Another point of difference is that the liver does not play such a dominant rôle in determining the depressor effect on intravenous injection as is the case with peptone or anaphylactic shock.

In general the results raise the question as to whether the active substance in peptone, tubocurarine or the substances used by MacIntosh and Paton act at a similar point in the cell. It is interesting to note here that various observers (see McIntyre, 1949) failed to obtain the expected response to peptone in curarized animals. The results also raise the question whether at some stage in the chain of events which follows the union of antibody and antigen a substance is produced which acts in a similar way.

SUMMARY.

Histamine is liberated from the isolated perfused lungs of the guinea-pig by d-tubocurarine. The pattern of output which may be interpreted as a diffusion process, resembles that caused by peptone or anaphylactic antigen.

Histamine and heparin are liberated from the dog's liver *in vivo* which becomes, after one or two injections, insensitive to further injections of tubocurarine but remains sensitive to histamine. *In vitro* only small amounts of histamine are liberated from the dog's liver into the perfusion fluid (Tyrode or heparinized blood).

The depressor effect of tubocurarine injected into the portal circulation is associated with obstruction of the hepatic venules caused by the liberation of histamine from the liver. When the drug is injected into a systemic vein the fall of blood pressure is not mainly due to the release of histamine from the liver, nor to a direct cardiac action, nor to pulmonary vascular obstruction. It is due to peripheral vasodilatation probably caused mainly by the liberation of histamine and partly by ganglionic block.

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THE PLASMA AND BLOOD VOLUMES OF NORMAL AUSTRALIAN MALES AND NOTES ON THEIR ESTIMATION

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The dye-dilution principle for the estimation of blood volume has been variously employed of recent years following a report of Gibson and Evans (1937) which showed the adaptability of T-1824 (Evans Blue) for this purpose. This seeming adaptability is the resultant of dye colour (Gregersen, 1937–38), the combination of the dye with plasma albumin in the form of a dye-protein complex (Rawson, 1943) and the relatively slow passage of this complex from the circulation (Gregersen and Rawson, 1943).

The investigation of the plasma volume, employing this procedure, in normal Australian males has not been previously undertaken at length. The following work is part of such an investigation.

METHODS.

General Procedure.

The subjects investigated were all apparently normal adult males—medical students, laboratory workers and hospital outpatients. The clothed body weight was directly measured with the subject wearing minimal clothing. The estimated or actual weight of this clothing was subtracted to give body weight.

Physically no subject was obese. Muscle development for the group was average. Body weight in no case was recently variable and no subject was recently convalescent from illness.

All subjects were fasting from 8–10 p.m. of the previous night—the last meal was at 6–7 p.m. Minimal water to drink was allowed in the fasting period.

Each separate investigation was commenced at 8.30–9.30 a.m. The subject had rested loosely in a nearly recumbent position for 20–30 minutes previously. This position was then maintained throughout the experiment.

T-1824.

The sample first used was Imperial Chemical (Pharmaceuticals) Ltd. Evans Blue. Latterly, British Drug Houses Ltd. Evans Blue (Laboratory Reagent) was used. This dye, by a polarographic technique, was shown to bind with plasma proteins. Such dyed plasma did not diffuse through a cellophane membrane at pH 7·2. The I.C.I. T-1824 in serum had a maximal light absorption at a wavelength of 620 mμ and followed Beer's Law (Isbister and Steinbeck, 1946).

The dye was dissolved as a 0·5 p.c. aqueous solution, autoclaved within glass ampoules and stored in the dark. Autoclaving did not affect the optical density of the solution.

The concentration of T-1824 within the plasma was directly estimated in the serum. The serum was doubly centrifuged. Optically, serum was clearer than plasma of particles and there is no evidence that T-1824 is withdrawn from solution in the clotting process (Gregersen and Schiro, 1938). The volume changes due to fibrin formation and contraction are within the overall error of the method. The net optical density of T-1824 in serum was determined with a "Spekker Photoelectric Absorptiometer" (Adam Hilger, Ltd.). This instrument was fitted with a single adaptor for 0·5 ml. capacity cells. In addition, the galvanometer was replaced by a critically damped string galvanometer reflecting on to a metre scale. The optical filter was a Wratten 29F combined with a Calorex type heat filter. This combination had a maximum transmission, 48·2 p.c., at a wavelength of 646 mμ. Below 600 mμ there was no light transmission. This did not exclude the non-specific density effect of haemolysis. The curvilinear calibration curve for the dye concentration range of 1·0–20·0 mg./l. was corrected for each serum by a microdilution technique (Isbister and Steinbeck, 1946).

Experimental Procedure.

In the estimation of plasma volume an intravenously indwelling, citrated lumbar puncture needle was inserted into an arm vein of one side. Through this, after a period, were withdrawn the blank sample—for photometric, haematocrit and plasma protein estimations—and serial samples after the dye injection. The dye was injected from a minimally paraffined, recalibrated all-glass syringe into a contralateral arm vein. The volume of dye injected, according to body weight, was 3–5 ml. Eight serial samples were taken between 10–52·5 min. after the dye injection. In these samples T-1824 concentration was estimated; in some the specific gravity of serum by the method of Phillips *et al.* (1945) was estimated and in others the haematocrit was checked. The dilution of dye that would obtain on injection if an instantaneously complete dilution of the dye with its final volume of distribution had occurred without loss of dye was derived by extrapolation. Extrapolation was performed on a semilogarithmic plot following Gregersen and Rawson (1943). Thus, the volume of the dye distribution or the plasma volume was calculated:

Plasma volume = amount of dye injected/hypothetical instantaneous concentration of dye.

Haematocrit.

The haematocrit value was obtained for blood oxalated with 6 mg. ammonium oxalate plus 4 mg. potassium oxalate per 5 ml. blood (Heller and Paul, 1934). This blood was centrifuged in duplicate within Wintrobe tubes at 3,000 r.p.m., at a 15 cm. radius, for 1 hour. This value was taken as 1·04 × actual haematocrit and empirically corrected to unity. This systematic correction is suggested by the results of Gregersen and Schiro (1938) and Shohl and Hunter (1940–41). Actual haematocrit × 0·95 as an empirical correction would fit their data more accurately. Duplicate values checked closely.

Blood Volume.

The apparent blood volume was derived by proportion with the corrected haematocrit.

RESULTS.

The Rate of Disappearance of the Dye from Plasma.

For a representative group of subjects the decreasing serum concentrations of T-1824 are shown as a linear plot against time in Fig. 1A and as a semilogarithmic plot in Fig. 1B. This latter graph reduced the curvilinear relation of the earlier one to rectilinearity. These time relationships are referred to as disappearance curves.

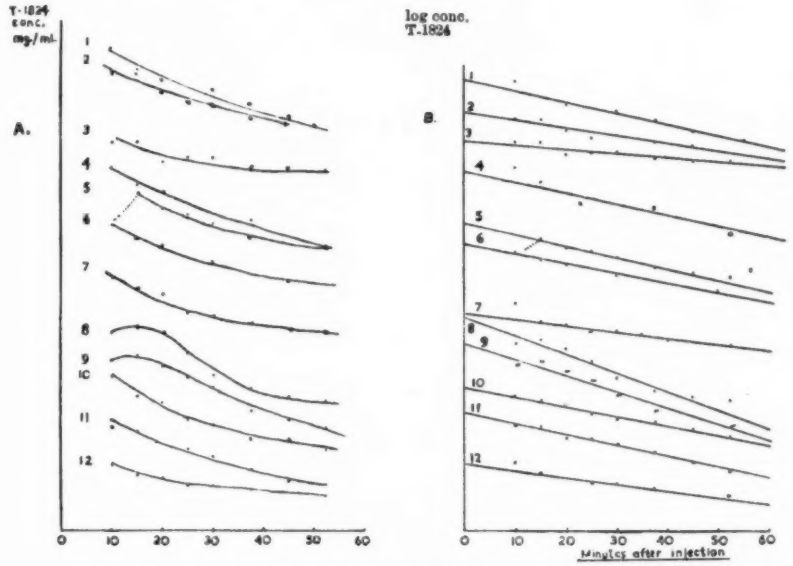

Fig. 1. Linear and semilogarithmic disappearance curves for T-1824 plotted with equal ordinates.

Such curves were plotted individually. Over the time range of sampling these curves did not significantly depart from the general pattern of an exponential growth curve, though this was most certain for the first 30 minutes after injection. The effective mixing of the dye with the volume of dilution was complete within 10 minutes after injection for 23 cases (72 p.c.), 10–15 minutes for 7 (22 p.c), less definite in the remaining 2.

The mean serum concentration of T-1824 at 30 minutes after injection was $93{\cdot}6 \pm 0{\cdot}48$ p.c. of the hypothetical initial concentration. The range was 85·7–97·6 p.c. with a mean square of 7·94 and 66 p.c. cases were between 90–95 p.c.

The Estimation of Plasma Volume.

The estimations of plasma volume for the respective subjects, detailed with respect to age and body weight, are listed in Table 1 and shown as a scatter diagram in Fig. 2.

For plasma volume and body weight the coefficient of correlation (r) was $+ 0{\cdot}6116^{**}$ (* = significant at 5 p.c. and ** = significant at 1 p.c. probability (P) level). The variance ratio (F) of plasma volume in between groups/within groups of body weight was $4{\cdot}550^{**}$ (8/23 d.f.). The linear regression coefficient (b) for plasma volume upon body weight was $0{\cdot}03259$ ($b/s_b = 4{\cdot}234^{**}$). The corresponding linear regression equation was:

$$\hat{Y} = 3{\cdot}439 + 0{\cdot}03259\ (X - 72{\cdot}81),$$

and $Sd_{y.x}{}^2 = 4{\cdot}506$, $s_{y.x} = 0{\cdot}3875$ l.

The variance ratio of plasma volume between groups of body weight in linear regression/deviations from regression was $15{\cdot}51^{**}$. Curvilinear regression was improbable as variance ratio of plasma volume in deviations from linear regression/within groups of body weight was 1·616 ($P \simeq 20$; 7/23 d.f.).

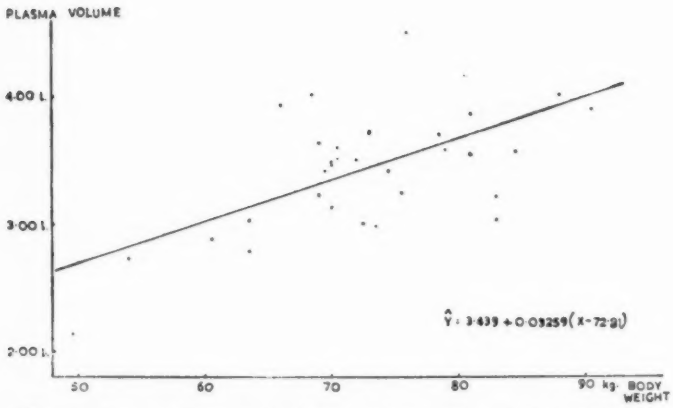

Fig. 2. Scatter diagram of plasma volume against body weight with the corresponding linear regression equation.

TABLE 1.

Subject	Age (yr.)	Wt. (kg.)	Plasma volume (l.)			Haematocrit
			Usual	Suggested methods	10 min. sample	actual × 1·00/1·04 (p.c.)
1	22	90·5	3·91	3·90	3·95	41·1
2	21	88·0	4·02	3·99	4·12	40·0
3	48	83·0	3·03	3·01	3·06	41·3
4	46	70·5	3·60	3·52	3·62	44·6
5	33	83·0	3·22	3·17	3·32	45·0
6	33	70·0	3·49	3·46	3·56	42·8
7	22	68·5	4·02	4·02	4·04	41·3
8	21	66·0	3·94	3·98	4·08	41·6
9	22	78·5	3·71	3·71	3·79	46·1
10	21	81·0	3·87	3·90	3·92	45·9
11	21	75·5	3·25	3·21	3·35	44·0
12	21	63·5	2·78	2·76	2·83	45·7
13	20	79·0	3·59	3·52	3·73	43·7
14	20	80·5	4·17	4·08	4·31	42·3
15	22	81·0	3·55	3·61	3·69	44·7
16	31	60·5	2·88	2·87	3·05	49·2
17	23	69·0	3·64	3·61	3·64	41·3
18	28	72·0	3·51	3·46	3·63	42·3
19	21	49·5	2·14	2·13	2·16	43·7
20	21	63·5	3·03	3·00	3·06	42·6
21	20	74·5	3·42	3·38	3·48	41·4
22	22	70·0	3·13	3·13	3·36	41·3
23	22	72·5	3·00	2·99	3·06	44·2
24	21	70·0	3·47	3·46	3·55	40·4
25	22	69·5	3·42	3·57	3·38	41·9
26	20	70·5	3·52	3·47	3·57	39·9
27	22	73·5	2·98	2·91	2·90	37·9
28	20	84·5	3·57	3·57	3·78	43·7
29	21	69·0	3·23	3·22	3·33	41·1
30	18	76·0	4·51	4·47	4·56	41·1
31	54	73·0	3·72	3·75	3·89	40·7
32	19	54·0	2·73	2·71	2·88	41·6

The plasma volume was also estimated from the disappearance rate of T-1824 in the first 30 minutes after injection. These estimates are shown parallel to the original figures in Table 1. Considered as two series of concomitant interpretations there was no significant difference between the group means ($d/s_d = 0{\cdot}132$, $d = \bar{x}_1 - \bar{x}_2$). These latter estimates were, however, less than the original in 23 instances and identical in five.

In addition, the plasma volume was calculated from the serum concentration of T-1824 in the sample taken 10 min. after injection (Table 1). In 30 instances these figures were in excess of the initial values but there was no significant difference between the group means ($d/s_d = 0{\cdot}665$).

The Estimation of Blood Volume.

The values derived from plasma volume and corrected haematocrit had a significant correlation with the body weight, $r = +0{\cdot}6040^{**}$. The linear regression coefficient for blood volume upon body weight was 0·05470 ($b/s_b = 4{\cdot}147^{**}$) so that $\hat{Y} = 5{\cdot}999 + 0{\cdot}05470\ (X - 72{\cdot}81)$ and $Sd_{y.x}{}^2 = 4{\cdot}506$, $s_{y.x} = 0{\cdot}6644$ l. However, the variance ratio of blood volume in between groups/within groups of body weight was 1·664 ($P \simeq 0{\cdot}17$; 8/23 d.f.).

The frequency distribution of plasma and blood volume did not show any reported value to be without the expected normal distribution. Values for Subject 30 are on the upper limit of normality.

The Plasma Volume Relative to Body Weight.

These derived values (v. Table 1) did not correlate significantly with the body weight ($r = -0{\cdot}2957$; $b/s_b = 1{\cdot}696$; $P = 0{\cdot}10$). Also, the variance ratio of relative plasma volume in within groups/between groups of body weight was 1·259 ($P > 0{\cdot}20$). The second degree regression for the same relationship had a multiple correlation coefficient (R) of 0·440*. For this regression, the variance ratio of relative plasma volume in regression/errors of estimate was 3·477* but the corresponding b primes were not significant.

The Plasma Volume Adjusted to 70 kg. Body Weight.

Adjustment was obtained with the linear regression equation. For the series mean = 3·347, mean square = 0·1417.

DISCUSSION.

The principle followed has been that the dye dilution represents a functional estimate of plasma volume under the conditions of the investigation. Direct estimation of the dye in serum has limited the method to the post-absorptive state. As T-1824 is not removed in the clotting process "serum volume" should not differ significantly from plasma volume. Nitshe and Cohen (1947) found the serum volume to average 5 p.c. less than the plasma volume. In a smaller unreported series the difference does not appear so great.

The time for complete mixing of the dye, now dye-albumin complex, with its volume of dilution and the rate of disappearance from this volume have been discussed by various investigators. The disappearances curves from this investigation suggest a normal variation in mixing time up to 15–20 minutes after injection. This is a reason for serial sampling up to 50–60 minutes after injection. On a semilogarithmic plot these curves have shown that rectilinearity is maintained most closely for 20 minutes after mixing. In estimation the

values derived by using only this fraction of the curve are not significantly different from the original values, though suggesting smaller terms. It is considered because of this that the latter method may prove the more accurate. The shorter time will obviate significant re-entry of T-1824 from the right lymph and thoracic ducts. In the anaesthetized dog and goat (Courtice, 1943) this re-entry is not significant for the first 45 minutes or so. Overbey *et al.* (1947) suggested an overall relationship for dye loss of log. concentration—log. time for 1 minute to about 60 minutes after the dye injection. The initial dye concentrations from such extrapolations tend to be less than those from the first 30 minutes of the semilogarithmic plot for the present series. This leads to a corresponding increase in the estimate.

Davis (1942) suggested that the 10 minute serum T-1824 concentration will allow an adequate estimation of the plasma volume. Values obtained by this method are not significantly different as a series from the initial values but are in excess of the latter in 30 instances. Basically, the tendency will be toward an overestimation of plasma volume in which direction the inherent errors of the method appear to lie (Cardozo, 1940-41; Courtice, 1943; Lawson *et al.*, 1947).

Linear regression of plasma volume upon body weight affords an adequate expression of the data. This function will be of value in forecasting. However, for an estimated individual plasma volume the standard error will be $\pm (0{\cdot}1549 + 0{\cdot}00005925x^2)^{\frac{1}{2}}$, where $x = X - 72{\cdot}81$. The 0·95 fiducial interval, $2{\cdot}042 \times$ S.E., refers to the difficulty of forecasting individual plasma volumes at the extremes of the range in the presence of wide individual variation.

Comparable figures reported by Gibson and Evans (1937), considering only subjects detailed by weight and height, on analysis gave $r = +0{\cdot}7082^{**}$; F (plasma volume in between/within groups weight) $= 3{\cdot}224^{**}$ (10/32 d.f.); $b = +0{\cdot}03360$ ($b/s_b = 6{\cdot}423^{**}$); $\hat{Y} = 2{\cdot}940 + 0{\cdot}03360$ $(X - 69{\cdot}10)$ and $s_{y.x} = 0{\cdot}3289$. If both series are adjusted to 70 kg. body weight the Australian mean is significantly greater than the latter ($d = 0{\cdot}385$, $s_d = 0{\cdot}08422$, $t = 4{\cdot}571^{**}$ with homogeneous variances, $F = 1{\cdot}199$, 31/42 d.f.). Correlation and regression coefficients are not significantly different.

Similarly, values published by Ling and Sprinz (1948, excluding the last subject) on analysis gave $r = +0{\cdot}6415^{**}$; $b = +0{\cdot}03590$ ($b/s_b = 4{\cdot}656^{**}$); $\hat{Y} = 3{\cdot}185 + 0{\cdot}03590$ $(X - 70{\cdot}12)$ and $s_{y.x} = 0{\cdot}3718$. For adjusted series the Australian mean is significantly greater than the latter ($d = 0{\cdot}197$, $s_d = 0{\cdot}09701$, $t = 2{\cdot}031^{*}$ with homogeneous variances, $F = 1{\cdot}125$, 32/31 d.f.). Again, the correlation and regression coefficients are not significantly different.

The results of Gibson *et al.* (1946), which are significantly different from those of Gibson and Evans (1937), when analysed give $r = +0{\cdot}6909^{**}$; $b = +0{\cdot}03743$ ($b/s_b = 5{\cdot}890^{**}$); $\hat{Y} = 3{\cdot}552 + 0{\cdot}03743$ $(X - 74{\cdot}43)$ and $s_{y.x} = 0{\cdot}3513$. For adjusted series there is no significant difference between means ($d = 0{\cdot}039$, $s_d = 0{\cdot}09108$, $t = 0{\cdot}428$ with homogeneous variances, $F = 1{\cdot}278$, 39/31 d.f.). Correlation and regression coefficients are not significantly different.

The report of Courtice and Gunton (1949) (by averaging body weight and plasma volume for the same individual, i.e. a series of 12) on analysis gave $r = +0{\cdot}9290^{**}$; $b = +0{\cdot}03359$ ($b/s_b = 7{\cdot}945^{**}$); $\hat{Y} = 2{\cdot}814 + 0{\cdot}03359$ $(X - 69{\cdot}63)$ and $s_{y.x} = 0{\cdot}1418$. For adjusted series the Australian mean is significantly greater than the latter ($d = 0{\cdot}521$, $s_d = 0{\cdot}1130$, $t = 4{\cdot}611$, with disparity in variances $F = 11{\cdot}52^{**}$, 31/11 d.f.). The correlation and regression coefficients do not differ.

These comparisons suggest that representative results for the estimation of normal plasma volume may fall into at least two groups—low and high value. The former is represented in the results of Courtice and Gunton (1949), the latter in the results of the present series and those of Gibson *et al.* (1946). The figures of Ling and Sprinz (1948) are intermediate. This grouping requires analysis of cause.

The plasma volume may also be expressed as ml./kg. body weight. This expression is not significantly correlated with body weight at the 5 p.c. level of probability ($r = -0{\cdot}2957$) and the variation between individuals is greater than that within groups. Graphically, a curvilinear relation of relative plasma volume with weight is suggested but the second degree regression analysis did not favour this relationship—the statistics are insufficient. The values of Gibson and Evans (1937) tested showed a correlation ratio just at the chosen level of probability significance (5 p.c.) with the volume variation within groups greater than between groups. Similar conclusions with respect to relative blood volume were also drawn. For the above weight levels, then, a curvilinear relationship of volume is improbable, though higher body weights are associated with lower relative volume. Any discussion of an optimal volume requires a greater body weight coverage in subjects.

TABLE 2.

The plasma volume, ml./kg., of normal male subjects determined by the T-1824 method.

Author	Number of subjects	Plasma volume (ml./kg.)	Mean square
Courtice and Gunton (1949)*	12	40·63	4·49
Crooke and Morris (1942)	5	49·78	5·80
Davis (1942)	11	39·97	25·74
Gibson and Davis (1937)*	43	42·77	27·92
Gibson *et al.* (1946)	40	47·91	30·02
Griffin *et al.* (1945)	59	36·72	—
Harington *et al.* (1940)	11	47·41	33·79
Hopper *et al.* (1944)	5	46·42	29·87
Ling and Sprinz (1948)	33	45·61	30·21
Mather *et al.* (1947)	53	54·80	48·72
Nitshe and Cohen (1947)	25	39·68	32·89
Noble and Gregersen (1946)	51	44·7	24·01
Root *et al.* (1946)	8	48·19	56·95
Steinbeck	32	47·44	30·90
Stewart and Rourke (1941)	11	45·66	42·79

* see text.

Available results, expressed as plasma volume relative to weight, were compared. The respective weight distributions are comparable. Table 2 summarizes the distribution. The range is probably 36·9–49·8 ml./kg. as Bowler *et al.* (1947) averaged falling plasma T-1824 concentrations. The present mean figure $47{\cdot}44 \pm 0{\cdot}98$ is significantly in excess of all values less than 45·00 ml./kg. Comparisons are shown in Table 3.

TABLE 3.

The comparison of the plasma volume, ml./kg., of normal male subjects with the present series.

Author	Difference between means (ml./kg.)	Standard error of difference	*t*	Variance ratio
Courtice and Gunton (1949)	6·81	1·632	4·178**	6·89**
Davis (1942)	7·47	1·879	3·973**	1·20
Gibson and Evans (1937)	4·67	1·252	3·736**	1·11
Gibson *et al.* (1946)	0·47	1·398	0·336	1·03
Harington *et al.* (1940)	0·03	—	—	1·09
Ling and Sprinz (1948)	1·83	1·360	1·346	1·02
Mather *et al.* (1947)	7·36	2·089	3·522**	1·58
Nitshe and Cohen (1947)	7·76	1·491	5·208**	1·06
Noble and Gregersen (1946)	2·74	1·336	2·362*	1·29
Root *et al.* (1946)	0·75	2·335	0·321	1·84
Stewart and Rourke (1941)	1·78	2·009	0·886	1·39

Both methods of comparison show that the present figures are significantly in excess of some reported values in the English and American literature. Attributing this variation to either physiological or technical features is at once difficult. The two series of Gibson—Gibson and Evans (1937); Gibson *et al.* (1946)—are significantly different with respect to adjusted means and relative volume though having a common method. A T-1824 variation due to a non-blue impurity might require consideration as Hartwell *et al.* (1936) noted a red impurity in the process of synthesis. An innate physiological variation due to climatic factors is not discernible from the present literature. However, a small number of unreported cases have winter figures less than summer figures. The position probably reflects some variation in technical details. This requires reconsideration of the statement of Gibson *et al.* (1946) that modern modifications of the dye dilution technique have clearly shown that plasma volume can be measured with a high degree of accuracy in normal men and that values for plasma volume in man determined by several workers are in general agreement both as to absolute plasma volume and plasma volume per unit of body measurement. In this respect the significantly small mean square for the results of Courtice and Gunton (1949) is to be noted.

The discussion of plasma volume applies to blood volume. Blood volume results appear less homogeneous than those for plasma volume. This may reflect the possibility that the corrected peripheral haematocrit is not a measure of or in constant relationship to body haematocrit.

In application, the variation of plasma volume will be best noted in relation to a previous estimate unless the body weight is near the mean of the series of derivation for the regression formula.

SUMMARY.

The plasma volume has been determined in healthy men by the T-1824 method. Blood volume has been derived with the haematocrit proportion.

The relationships of plasma, blood volume and body weight have been considered.

The comparison of reported values for plasma volume has been discussed.

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MIXED BACTERIAL GROWTH

II. THE GROWTH OF *STAPH. AUREUS, STR. PYOGENES* AND A VIRIDANS STREPTOCOCCUS IN MIXED BROTH CULTURES

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In a previous publication (Annear and Hayes, 1950) a technique was described for the enumeration of viable cells of *Staph. albus* and a viridans streptococcus from mixed broth cultures. Selective media containing sulphanilamide (SA) and crystal violet (CV) were used for the recovery of the staphylococcus and streptococcus respectively. In this paper, application of the technique to the study of further strains of staphylococci and streptococci in mixed broth cultures, is described. Sulphanilamide, although satisfactory, was not ideal for this type of work and in the following experiments has been replaced by sodium chloride.

EXPERIMENTAL.

Apart from the substitution of sodium chloride for sulphanilamide, the materials and the methods used for growth curve control and growth curve experiments were identical with those described in detail by Annear and Hayes (1950).

Chapman (1945) observed that staphylococci were resistant to 7·5 p.c. sodium chloride in agar whereas most other bacteria were susceptible. The concentration of salt[1] in agar, most suitable for the recovery of staphylococci and inhibition of streptococci was ascertained by comparing the growth of numerous strains of *Staph. albus, Staph. aureus, Str. pyogenes* and viridans streptococci in agar containing from 2·5–7·5 p.c. salt. Five p.c. salt allowed maximum recovery, as compared with nutrient agar, of all strains of staphylococci and inhibited all strains of streptococci tested. Thirty strains of staphylococci were tested and found to be uniformly resistant to salt (5 p.c.) and inhibited by CV (1:1,000,000) in agar. The opposite was the case with thirty strains of streptococci tested.

Comparative tests for the efficiency of salt and SA agar as recovery media for *Staph. albus* in the presence of the viridans streptococcus (strains used in previous experiments) showed salt agar to be a more effective medium. In salt agar (5 p.c.) the staphylococcus could be recovered in the presence of approximately 15×10^6 viable viridans streptococci; in SA agar (1:3,000) the presence of more than approximately 1×10^6 viable viridans streptococci interfered with the recovery of the staphylococcus. In salt agar there was no satellitism of streptococcal colonies around staphylococcal colonies as there occasionally was in SA agar.

[1] Salt refers to sodium chloride.

The strains of *Staph. aureus* and *Str. pyogenes* chosen for the following experiments were selected at random from those strains tested against salt and crystal violet.

The strain of viridans streptococcus used was selected because of knowledge gained of its behaviour in previous experiments (Annear and Hayes *loc. cit.*). The strain of *Staph. aureus* produced haemolysis on horse blood agar, was coagulase positive and pathogenic for rabbits. In 5 p.c. salt agar, *Staph. aureus* could be recovered in the presence of approximately 150×10^6 viable viridans streptococci and approximately 50×10^6 viable cells of *Str. pyogenes*. In CV (1:1,000,000) agar the viridans streptococcus and *Str. pyogenes* could be recovered in the presence of approximately 4×10^6 viable cells of *Staph. aureus*. The necessary growth curve control experiments were done before each growth curve experiment was undertaken. In the mixed cultures studied, the strain of *Staph. aureus* was grown in the presence of (1) the viridans streptococcus and (2) *Str. pyogenes*.

Staph. aureus *and the Viridans Streptococcus.*

Several mixed growth curve experiments were done, varying the initial inoculum of the two organisms. Only one of the mixed growth curve experiments is illustrated here (Fig. 1). The behaviour of the staphylococcus and viridans streptococcus in the mixture during the first 48 hours of growth was influenced largely by the initial ratio of the two organisms. If the broth was inoculated with approximately equal numbers of the two organisms no suppression of either staphylococcus or streptococcus took place. If, in the initial inoculum, the streptococcus greatly exceeded the staphylococcus, the growth of the latter was suppressed during the first six hours. Normal growth followed after this initial suppression, counts reaching their maximum about six hours later than the control. In the presence of large numbers of staphylococci, the viridans streptococcus was suppressed after 8 hours, the number of viable cells never equalling those recovered from the pure culture controls (Fig. 1).

After 48 hours the growth curves for both organisms appeared to follow a persistent pattern, independent of the initial ratio at 0 hours and the type of curve produced in the first 48 hours. Fig. 1, representing a mixed growth curve experiment carried on for 35 days, illustrates these patterns. The staphylococcus control curve, which closely resembles that of the mixture, has been omitted to avoid confusion of the graph. In the mixture the staphylococcus remained in the stationary phase, falling slightly, while the viridans streptococcus produced an undulating curve until 24 days, then gradually decreased in numbers and could not be recovered after 35 days. In the pure culture control the viridans streptococcus curve appeared as a series of much deeper undulations. The organism was viable at 27 days but counts were discontinued because of contamination. The height of the viridans streptococcus curve from the mixture, as compared with the control, suggests that the staphylococcus was exerting some stimulating effect.

Staph. aureus *and* Str. pyogenes.

Only one mixed growth curve experiment was done with the above organisms, equal inocula being used. The experiment was done in duplicate, the resulting growth curves being almost identical. The staphylococcus curve from the mixture is similar to that shown in Fig. 1; the control curve is omitted from the graph to avoid confusion. The growth curve from the control culture of *Str. pyogenes* showed no stationary phase, the decline phase setting in almost immediately after the maximum cell concentration was reached (Fig. 2). The decline was gradual, the culture being viable but in greatly reduced numbers at 16 days, when the experiment was discontinued. In the mixture, the streptococcus was slightly suppressed at 10 hours, and the curve did not reach the height of the control. The decline, however, was more gradual in the

mixture, the curve flattening out at approximately 7 days and remaining so until the experiment was discontinued. At 16 days, the streptococcus count of the mixture was approximately 1,000 times greater than that of the control culture, suggesting that the staphylococcus was supporting the growth of the streptococcus in the mixture.

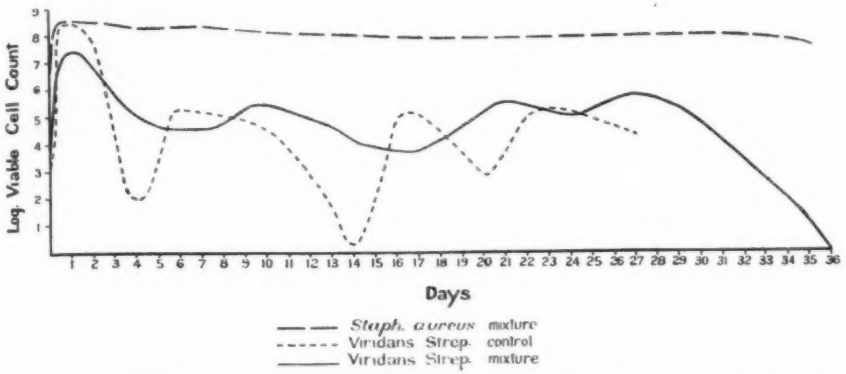

Fig. 1. Growth curves illustrating the behaviour of *Staph. aureus* and the viridans streptococcus in mixed broth culture, and the viridans streptococcus in pure culture (control).

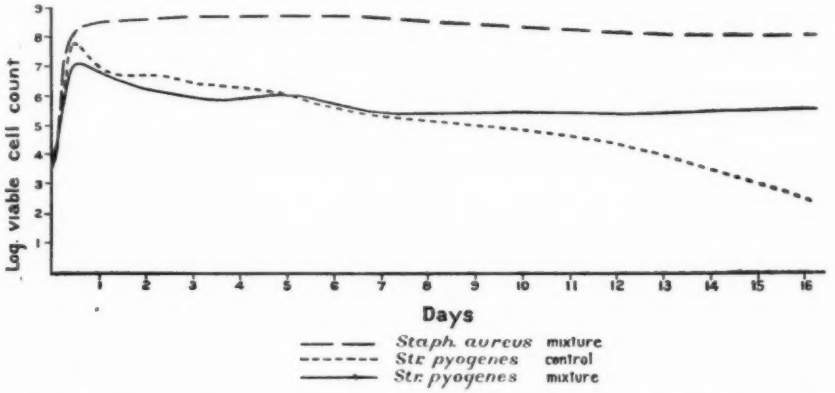

Fig. 2. Growth curves illustrating the behaviour of *Staph. aureus* and *Str. pyogenes* in mixed broth culture, and *Str. pyogenes* in pure culture (control).

Statistical Analysis of Viable Counts.

In previous work (Annear and Hayes, 1950) the streptococcal and staphylococcal counts were submitted to the χ^2 test and examined for conformation to Poisson distribution. The results of these analyses provided evidence for the reliability of the counts and the suitability of the various agar media employed. While the great majority of these counts showed variances that were to be expected there were excessive frequencies of counts showing both

unduly low ($P>\cdot99$) and high ($P<\cdot01$) variance. The excess of $P>\cdot99$ values arose from duplicate counts and the excess of values of $P<\cdot01$ arose mainly from the staphylococcal counts in sulphanilamide agar.

Similar χ^2 analyses were done with the triplicate counts in this investigation (no duplicate counts were made), and the Goodness of Fit P values from the resulting frequency distributions are shown in Tables 1 and 2. The counts showed no undue degree of either excessively low or excessively high variance. It is significant that the staphylococcal counts in salt agar showed no excess of values of $P<\cdot01$. The Goodness of Fit P values in Tables 1 and 2 are satisfactory, indicating the reliability of the counts, and showing that the replicate counts varied as small Poisson samples.

TABLE 1.

Goodness of Fit probability values from frequency distributions of observed and expected χ^2 *values of replicate colony counts of* Staph. aureus.

	Nutrient agar (1)	Salt agar, 5 p.c. (2)	Salt agar, Streptococci present (3)	Salt and nutrient agar (4)	Total (5)
Replicates	3	3	3	6	3
No. of counts	110	110	110	104	330
P (Goodness of Fit)	·86	·72	·33	·45	·64

(3) Addition of counts of *Staph. aureus* in 5 p.c. sodium chloride agar in the presence of the viridans streptococcus or *Str. pyogenes*.

(4) Parallel counts in the two media made from control cultures.

TABLE 2.

Goodness of Fit probability values from frequency distributions of observed and expected χ^2 *values of replicate colony counts of* Str. pyogenes.

	Nutrient agar (1)	Crystal violet agar 1:1,000,000 (2)	Crystal violet agar *Staph. aureus* present (3)	Crystal violet and nutrient agar (4)	Total (5)
Replicates	3	3	3	6	3
No.of counts	50	36	40	35	126
P (Goodness of Fit)	·07	·05	·38	·60	·20

(4) Parallel counts in the two media from control cultures.

The parallel counts of each pure culture made in nutrient and selective agars during the growth curves were tested for significant differences. The sets of 6 counts (3 in each medium) were examined by the χ^2 test for conformation to Poisson distribution (column 4 of Tables 1 and 2). In both cases the Goodness of Fit P values were satisfactory, showing that chance

differences only occurred among the counts, and that there were no significant differences between the two groups of triplicate counts, i.e. between (1) the recoveries of *Staph. aureus* in nutrient and in salt agars and (2) the recoveries of *Str. pyogenes* in nutrient and CV agars.

No results of statistical analyses of the counts of the viridans streptococcus have been shown, as evidence for the reliability of both nutrient and CV agars as recovery media for this organism was presented in a previous paper (Annear and Hayes, *loc. cit.*).

DISCUSSION .

No attempt has been made to explain the nature of the mixed growth curves. As the mixed cultures age, the staphylococcus appears to support the growth of the viridans streptococcus and the strain of *Str. pyogenes*. The difference in the nature of the curves produced by the two species of streptococci is of interest, but whether this is a persistent species difference or not, has yet to be ascertained.

The genus difference in susceptibility of staphylococci and streptococci (*Str. pyogenes* and viridans streptococci only; enterococci tolerate 5 p.c. salt) to crystal violet and sodium chloride in the concentrations used seems consistent. A large number of strains of staphylococci and streptococci would have to be tested before any dogmatic statement to this effect could be made.

The differences in behaviour of *Staph. albus* and *Staph. aureus* in the presence of the viridans streptococcus are of interest. In earlier experiments (Annear and Hayes, 1950) the growth of *Staph. albus* was suppressed by the viridans streptococcus except in growth curves where the initial inoculum of *Staph. albus* greatly exceeded that of the viridans streptococcus. The viridans streptococcus was not as active in suppressing the growth of *Staph. aureus*, suppression occurring only when the initial inoculum of the streptococcus greatly exceeded that of the staphylococcus. The presence of large numbers of *Staph. albus* stimulated the growth of the viridans streptococcus whereas the presence of large numbers of *Staph. aureus* suppressed the growth of the viridans streptococcus. In preliminary experiments with salt agar it was observed that *Staph. albus* could be recovered in the presene of approximately 15×10^6 viable viridans streptococci and that *Staph. aureus* could be recovered in the presence of 150×10^6 viable viridans streptococci. It is possible that the pathogenic *Staph. aureus* may liberate products which are toxic for the viridans streptococcus.

Mixed bacterial flora occur normally in the body, staphylococci and streptococci growing in close association in the nasopharynx. In conducting the above experiments it was felt that any information on the viability of such organisms *in vitro* would contribute to the present scanty knowledge of the behaviour of micro-organisms in mixed populations.

SUMMARY.

Differences in sensitivity of *Staph. aureus, Str. pyogenes* and a viridans streptococcus to crystal violet and sodium chloride in nutrient agar, enabled the enumeration of viable cells to be made on certain mixed cultures of the above organisms.

Crystal violet (1 : 1,000,000) inhibited the growth of the staphylococcus and allowed maximum recovery of the viridans streptococcus and *Str. pyogenes.* The opposite was the case with 5 p.c. sodium chloride in agar.

Statistical analysis of the counts obtained from the growth curve experiments provided evidence for the efficiency of the crystal violet and sodium chloride agars as recovery media.

Observations were made (i) on the survival of *Staph. aureus* and a viridans streptococcus in mixed broth culture over a period of 35 days and (ii) on the survival of *Staph. aureus* and *Str. pyogenes* in mixed broth culture over a period of 16 days.

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